Universidade de Aveiro Departamento de Biologia
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Genotoxicity and Cytotoxicity of Cr(VI) and Pb^{2+} in *Pisum sativum*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutoramento em Biologia, realizada sob a orientação científica da Doutora Maria da Conceição Lopes Vieira dos Santos, Professor Associada com Agregação do Departamento de Biologia da Universidade de Aveiro e do Doutor John Williams Mundy, Professor e Vice-reitor da Faculdade de Ciências da Universidade de Copenhaga.

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Aos meus Pais e a Raquel.

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Palavras-chave

Ciclo Celular, Citometria de Fluxo, Citotoxicidade, Chumbo, Cloroplastos, Crómio, Ensaio Cometas, Dano DNA, Enzimas do Ciclo de Calvin, Fotossíntese, Genotoxicidade e *Pisum sativum*

Resumo

A toxicidade dos metais é uma problemática que envolve a saúde humana e o ambiente, sendo necessária uma vigilância constante e uma avaliação dos danos precisa e robusta. As plantas, como principal fonte alimentar e de produtos, são de vital importância à sociedade humana. Devido a serem seres sesseis, este grupo é um dos mais afectados por poluentes, tornando-os objectos de estudo extremamente interessantes.

O objectivo desta tese foi avaliar os efeitos genotóxicos e citotóxicos do Cr(VI) e Pb^{2+} na espécie modelo *Pisum sativum* L. No capitulo I é introduzida a problemática da toxicidade de ambos os metais, com especial relevo nas plantas, bem como as abordagens mais actuais no estudo da geno e citotoxicidade.

No capitulo II são apresentados os resultados dos estudos da genotoxicidade do Pb^{2+} (II-1) e Cr(VI) (II-2 e II-3), tendo sido realizados analises de dano ao DNA a vários níveis e alterações do ciclo celular (II-1 e II-2), bem como a detecção de instabilidade de microssatelites (II-1 e II-3), que é um indicador do estado funcional do mecanismo de reparação do DNA.

O capítulo III aborda o efeito de stresses abióticos na capacidade fotossintética da espécie modelo. No capítulo III-1, realizou-se um estudo pioneiro de avaliação da aplicabilidade da citometria de fluxo no estudo da fotossíntese, mais concretamente no estado funcional e estrutural dos cloroplastos, quando expostos a um inibidor da fotossíntese (Paraquat). Os dados obtidos neste estudo encorajaram a aplicação da técnica nos capítulos III-2 e III-3, nos quais se analisaram os efeitos dos metais Pb^{2+} (III-2) e Cr(VI) (III-3) na capacidade fotossintética de plantas expostas a este metal; em estudos que envolveram vários marcadores clássicos, para alem dos da citometria de fluxo.

Finalmente, no capítulo IV são apresentadas as conclusões finais do trabalho, uma comparativa entre os efeitos e níveis de toxicidade dos dois metais em estudo e são apontadas algumas perspectivas para futuros estudos, levantadas pelos dados obtidos.

Keywords

Calvin Cycle Enzymes, Cell Cycle, Chromium, Comet Assay, Chloroplasts, Cytotoxicity, DNA Damage, Flow Cytometry, Genotoxicity, Lead, Photosynthesys and *Pisum sativum*.

Abstract

Metal toxicity is both an environmental and human health concern that requires constant vigilance and precise and unbiased evaluation. Plants, as a major source of food and many goods are extremely important for society. Due to their sessile nature, these organisms are one of the groups which are more affected by pollution, being for this reason an exciting model group

The objective of this PhD thesis was the evaluation of the genotoxic and cytotoxic effects of Pb^{2+} and Cr(VI), in the model species *Pisum sativum* L.

In chapter I, the problematic effects of these metal's toxicities in plants is introduced, altogether with some of the most recent approaches to study geno and cytotoxicity.

Chapter II presents the results of the investigations regarding the genotoxicity of Pb^{2+} (II-1) e Cr(VI) (II-2 e II-3). To this end, an evaluation of the different levels of DNA damage and cell cycle dynamics were performed (chapters II-1 and II-2). Also, the induction of microsatellite instability, an indicator of the functional status of the DNA repair machinery, by this metals was also studied (II-1 and II-3)

The effect of abiotic stress at the photosynthetic level of this study's model species is reported in Chapter III. In chapter III-1, an innovative study was undertook, in which the applicability of flow cytometry to photosynthetic studies was evaluated, namely, in the evaluation of the structural and functional status of chloroplast exposed to a photosynthesis inhibitor (Paraquat). The results of that study led the way to the application of that technique in chapters III-2 and III-3, where the effects of Pb^{2+} (III-2) and Cr(VI) (III-3) in the photosynthetic status of plants exposed to this metal were evaluated, using classical markers, besides the ones established for flow cytometry in chapter III-1

Finally, in chapter IV, the final conclusions of this thesis are presented, as well as a comparative between the different effects and levels of toxicity of the two metals studied, together with some future perspectives in this field of research, based on the results presented throughout this thesis.

“You can’t always get what you want, but if you try you might find, you get what you need”

Mick Jagger

“Para comer guaraguara hay que mojarse el culo”

Eleazar Rodriguez Rojas

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CHAPTER I-

METAL PHYTOTOXICITY – A GENERAL INTRODUCTION

Chapter partially submitted as an invited book chapter:

Santos C and Rodriguez E (2011) Review on some emerging endpoints of Chromium (VI) and Lead phytotoxicity. In John Kiogora Mworia Editors. Botany. (in press).

Metal pollution: Facts and considerations

Metals occur naturally in the environment as constituents of the Earth's crust. They tend to accumulate and persist in the ecosystems due to their stability and mainly because they cannot be degraded or destroyed. However, and despite that in some cases (*e.g.* Mercury) high levels of metals may occur naturally; for most situations, anthropogenic activities are the primary cause for metal pollution. Examples of important sources of metal contamination come from industrial applications, mining, smelters, combustion by-products and fuel. From these sources, contaminants can enter the ecosystem as airborne particles, wastewaters and sludge, polluting sites near the source but also locations thousands of kilometers away. Actually, just by checking the news, the magnitude and reach of some catastrophic disasters can be observed, like the mass lead poisoning in Zamfara, Nigeria (March 2010) with more than 18000 persons affected or the toxic red sludge spilled from a aluminium production factory into the Danube river in Hungary (October 2010). Some cases, although more constricted, were far more famous, like the groundwater contamination of the town of Hinkley (California, USA) with hexavalent chromium, by the Pacific Gas & Electric company, which resulted in a legal case and multi-million dollar settlement (and also originated the 2000's Erin Brockovich movie). Moreover, studies like the ones of Murozumi *et al.* (1969), Hong *et al.* (1994) or McConnell and Edwards (2008) demonstrate the extension and persistence of metals in the environment. These studies also show that the contamination of the environment with these pollutants started way before the industrial revolution, like lead pollution originated by Roman mining and smelters in 500 B.C. (Nriagu, 1996).

Due to the above reasons and also to their toxicity to humans and environment, metal toxicity has become an increasing target of studies in humans, animals and plants. Of what is generally conceived, toxicity is originated through a very complex pattern of metal interactions with cellular macromolecules, metabolic and signal transduction pathways and genetic processes (Beyersmann and Hartwig, 2008).

Among the different models available to study metal toxicity, plants present some unique features that make them interesting subjects. Firstly, much of human diet depend directly from plants products like fruits and vegetables or indirectly as fodder given to livestock. Secondly, lacking the ability to escape from contaminated sites, plants evolved mechanisms to handle exposure to toxicants: from the amount that is taken from the surroundings; to sequestration and inactivation in sub cellular compartments; to tolerating the deleterious effects of metals. For instance, regarding the amount of pollutant accumulated, three categories of plants were proposed by Baker (1981): (1) excluders- those that grow in metal-contaminated soil and maintain the shoot concentration at low level up to a critical soil value above which relatively unrestricted root-to-shoot transport results; (2) accumulators- those that concentrate metals in the aerial part, and (3) indicators- where uptake and transport of metals to the shoot are regulated so that internal concentration reflects external levels, at least until toxicity occurs.

The toxicity of metals and their compounds largely depends on their bioavailability, *i.e.* the mechanisms of uptake through the cell's membrane, intracellular distribution and binding to cellular

macromolecules (Beyersmann and Hartwig, 2008). Although the relative toxicity of different metals to plants can vary with plant genotype and environmental/experimental conditions, most act through one of the following: changes in the permeability of the cell's membrane; reactions of sulphydryl (–SH) groups with cations; affinity for reacting with phosphate groups and active groups of ADP or ATP; replacement of essential ions and oxidative stress (Patra *et al.,* 2004). Through these, some of the most common symptoms of metals phytotoxicity are: growth inhibition, nutrient imbalance, disturbances in the ion and water regime (Gyuricza *et al.,* 2010), photosynthetic impairment (Hattab *et al.,* 2009b) and genotoxicity (Monteiro *et al.,* 2010).

Among the elements referred to as "heavy metals", 13 have been considered by the European Union to be of the highest concern: Arsenic (As), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Mercury (Hg), Manganese (Mn), Nickel (Ni), Lead (Pb), Tin (Sn) and Thallium(Ti). From these, some have been target of many investigations (*e.g.* Cd) while for others, the level of knowledge about the mechanism of toxicity are highly unsatisfactory (*e.g.* Cr). Interestingly, despite being one of the first metals with known reports of human poisoning, not enough research have been undertaken to clarify Pb's mechanism of toxicity and even some conflicting data have been reported (García-Lestón *et al.,* 2010).

Knowing that there is still a lot of work left to be done regarding metal toxicity, the works which compose this thesis were planned and executed, in order to provide new insight and better comprehension on these metal's phytotoxicity. Being this, the next chapters of the introduction will be dedicated to elucidate the reader about the two metals studied in the work presented in this thesis, Chromium and Lead.

Chromium: the element

Chromium was discovered in 1797 as part of the mineral crocoite, used as pigment due to its intense coloration. As a matter of fact, the name chromium is derived from the Greek word "χρώμα" (chroma- color) due to that propriety of the element (Shanker *et al*., 2005)

Chromium is the 21st most abundant element in Earth's crust with an average concentration of 100 ppm, ranging in soil between 1 mg/kg and 3000 mg kg^{-1}; in sea water from 5 μg l^{-1} to 800 μg l^{-1} and in rivers and lakes between 26 μg l^{-1} and 5.2 mg l^{-1} (Kotas and Stasiska 2000). Normally, Cr is mined from chromate but native deposits are not unheard off.

One of the most interesting characteristics of this metal is its hardness and high resistance to corrosion and discoloration. The importance of these proprieties resulted among others in the usage of this metal in the development of stainless steel, which together with chrome plating and leather tanning, are the most important applications of this element and the main sources of Cr pollution of the environment.

Chromium is highly soluble under oxidizing conditions and forms, exhibiting a wide range of possible oxidation states (from -2 to +6), being that +3 and +6 are the most stable forms. Under reducing conditions, Cr(VI) converts to Cr(III) that is insoluble, but this form is strongly absorbed onto the surface of soil particles.

Chromium: Uptake and assimilation by plants

Chromium is a common contaminant of surface waters and ground waters because of its occurrence in nature, as well as anthropogenic sources (Babula *et al.,* 2008). The two most common and stables species, Cr(III) and Cr(VI) are also the obvious forms of Cr contamination.

The most important sources of Cr(III) are fugitive emissions from road dust and industrial cooling towers; Cr(VI) compounds are used in the manufacture of pigments, in metal-finishing and chromium-plating, in stainless steel production, in hide tanning, as corrosion inhibitors, and in wood preservation (Shtiza *et al*., 2008).

Very few studies have attempted to elucidate the plant's mechanism for Cr uptake (*e.g* Figure 1), but factors like oxidative Cr state or its concentration in substrate play important roles (Babula *et al.,* 2008). Of what is known, due to its higher solubility and therefore bioavailability, Cr(VI) is more toxic at lower concentrations than Cr(III), which tend to form stable complexes in soils (Lopez-Luna *et al.,* 2009). The pathway of Cr(VI) transport is thought to be an active mechanism involving carriers of essential anions such as sulfate (Cervantes *et al.,* 2001). Fe, S and P are also known to compete with Cr for carrier binding (Wallace *et al.,* 1976). Moreover, Cr absorption and translocation have been show to be modified by soil pH, organic matter content and chelating agents, among others (Han *et al.,* 2004).

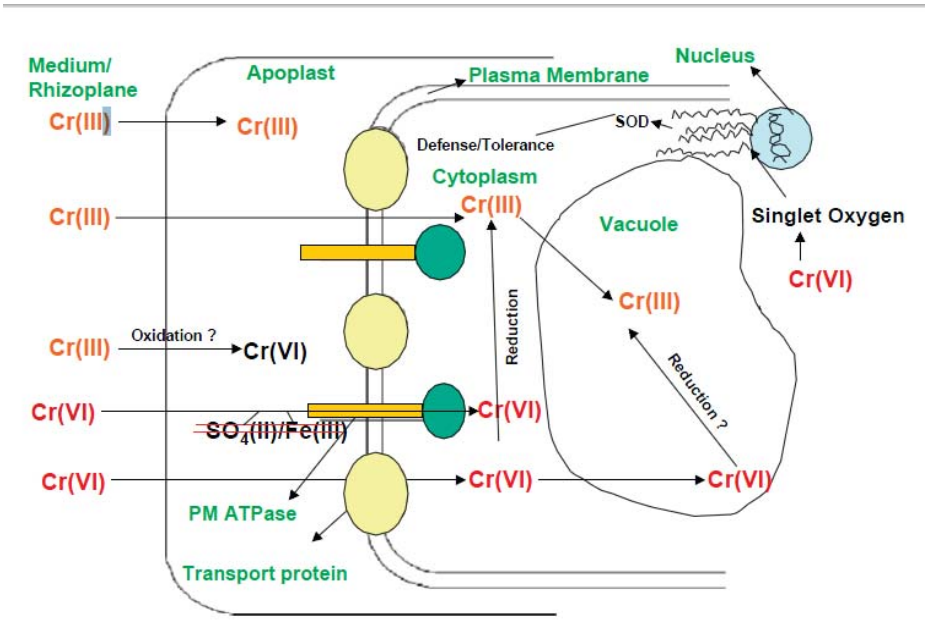

Figure 1 Hypothetical model for Cr transport and toxicity in roots. Taken from Shanker *et al.* (2005).

Studies performed to elucidate the uptake mechanism of Cr have demonstrated that only Cr(VI) is detected in plant tissues. However some plants (such as soybean and garlic) have the capacity to reduce Cr(VI) to unstable intermediate like Cr(V) and Cr(IV), or eventually to the more

stable form, Cr(III); this represents the detoxification pathway of Cr(VI) (Babula *et al.,* 2008). As this mechanism of detoxification is performed readily in the roots and as Cr is immobilized in the vacuoles of the root cells, the amount of Cr translocated to the aerial portion of the plants is very little (Shanker *et al.,* 2005).

In what concerns the methodological approaches applied in this thesis, the amount of Total Cr in the tissues was quantified instead of the amount of Cr(VI). This was due to the to the complex mechanism of Cr speciation in vivo, which despite very interesting was not a target of the research presented in this thesis.

Also, and despite that several studies on metal toxicity are performed in hydroponic cultures to avoid taking into account the interaction with soil constituents (complex matrix), the investigations reported in this dissertation were performed in soils contaminated with metal solutions of known concentration (Pb and Cr). This was decided in order to mimic, as closely as possible, the agricultural conditions in which important crops can be exposed to Cr/Pb by tainted water supplies. With this approach, it is expected that despite that part of the metals in the solution would not be available to the plants (probably due to complexation with other elements in the soil), these studies would provide data that could more easily predict the toxicity in real case pollution scenarios.

Chromium: Phytotoxicity

General effects

The effects of Cr in some of the classical endpoints of metal genotoxicity have received some attention by fellow researchers. Seed germination and plants growth are two of the parameters that have been studied thoroughly in several plants species. Results indicates that Cr provokes growth inhibition of roots in species like *Salix viminalis* (Prasad *et al.,* 2001), *Caesalpinia pulcherrima* (Iqbal *et al.,* 2001), wheat (Chen *et al.,* 2001) and mung bean (Samantaray *et al.,* 1998). Shanker *et al.,* (2005) hypothesize that the common root growth impairment observed in plants exposed to Cr could be due to inhibition of root cell division/root elongation or to the extension of cell cycle.

Aerial part growth has also been proven to be negatively affected by Cr in species like rice (Singh *et al.,* 2006) wheat, oat and sorghum (Lopez-Luna *et al.,* 2009), where the length of these shoots decreased as well as the number of leaves and their area. A justification to these facts was proposed by Shanker *et al.,* (2005), by stating that root growth inhibition, which leads to nutrient imbalance, could be behind low shoot development. Cr, due to its structural similarity with some essential elements, can affect mineral nutrition of plants in a complex way (Shanker *et al.,* 2005) and there has been innumerous considerations regarding this issue, especially in crops species. For instance, it has been demonstrated that very low concentrations of Cr (0.05–1 mg l^{-1}) promoted growth, increased nitrogen fixation and yield in leguminosae (*e.g.* Hewitt, 1953). At higher concentrations and just giving a couple of examples, authors have successfully proven that this

metal reduces the uptake of the essential elements Fe, K, Mg, Mn P, and Ca in *Salsola kali* (Gardea-Torresdey *et al.,* 2005) and K, Mg, P, Fe and Mn in roots of soybean (Turner and Rust, 1971). The justification of nutrient imbalance have been hinted as a competitive binding to common carriers by Cr(VI), the inhibition of the activity of plasma membrane's H^+ ATPase and the reduced root growth and impaired penetration of the roots into the soil due to Cr toxicity (Shanker *et al.,* 2005).

Photosynthesis

Like other metals, Cr can affect photosynthesis severally and in many different steps, which can ultimately translate in loss of productivity and death. Shanker *et al.* (2005), in a review about Cr phytotoxicity, discussed that while Cr toxicity at the photosynthetic level was well documented in trees and higher plants, the exact target and mechanisms affected by this metal were poorly understood.

Cr(VI) can easily cross biological membranes and has high oxidizing capacity, generating ROS which might induce oxidative stress (Pandey *et al.,* 2009). ROS are generated in normal metabolic processes like respiration and photosynthesis, being chloroplasts one of the main sites of reactive oxygen production and detoxification (Mittler, 2002). However, because of chloroplasts' complex and numerous membrane systems, rich in polyunsaturated fatty acids, this organelle might also be a target for peroxidation (Hattab *et al.,* 2009b) and one of the ways by which photosynthesis is affected. A common parameter affected by Cr is the amount of photosynthetic pigments, which tends to decrease when plants or algae are exposed to high doses of this metal (Vernay *et al.,* 2007, Subrahmanyam, 2008, Rodriguez *et al.,* 2011). The results obtained by Juarez *et al.,* (2008) demonstrated that, ROS caused structural damage to the pigment-protein complexes located in the thylakoid membrane (*e.g.* the destabilization and degradation of the proteins of the peripheral part of antenna complex), followed by the pheophytinization of the chlorophylls (substitution of Mg^{2+} by H^+ ions), and destruction of the thylakoid's membranes. It has also been demonstrated that Cr affects and might even inhibit pigment biosynthesis, among others, by degrading δ-aminolaevulinic acid dehydratase (Vajpayee *et al.,* 1999), an essential enzyme in chlorophyll biosynthesis. Vernay *et al.* (2007) also presented evidence that this metal probably competed with Fe and Mg for assimilation and transport to the leaves and therefore affected different steps of pigment biosynthesis.

Another endpoint of Cr phytotoxicity is Chl *a* fluorescence; however, some of the evaluation of some of the common biomarkers of Chl *a* fluorescence status demonstrated there is a certain level of resistance to Cr toxicity (namely the F_v/F_m). On the other hand, the ones related to the fluorescence emission status of light adapted-plants have been shown to be highly affected by this metal (Vernay *et al.,* 2007, Subrahmanyam, 2008). Several hypotheses explaining these results have been proposed, *e.g* structural alterations in the pigment–protein complexes of PSII or impairment in energy transfer from antennae to reaction centers (like a diversion of electrons from

the electron-donating side of PSI to Cr(VI) -Shanker *et al.,* 2005). Recently, Henriques (2010) implied that Cr(VI) might not be directly responsible for the damage to the chloroplast as the valence state of Cr depends of the local pH and redox values. For instance, in irradiated chloroplasts, physiological conditions would favor the less toxic Cr(III) form over the highly toxic Cr(VI). Appenroth *et al.,* (2000) demonstrated that Cr damaged the water oxidizing centers (WOC) associated to PSII and Henriques, (2010) proposed that this could be explained by the reduction of Ca's and Mn's availability (caused by Cr), being that these elements are fundamental in the structure and functioning of the WOC.

Besides the photochemical process, Cr is also known to cause distress in the biochemical aspects of photosynthesis. Vernay *et al.* (2007) and Vernay *et al.* (2008) discussed that despite the loss of biomass and wilting were common symptoms of Cr exposure little was known about Cr effect on water status and gas exchange. Subrahmanyam (2008) commented that it was unclear if Cr-induced inhibition of the photosynthetic process was also due (among others previously mentioned factors) to Cr-induced interference with the Calvin cycle's enzymes. In those reports, the authors proved that Cr consistently affected parameters like *E* (transpiration rate), g_s (stomatal conductance), *A* (photosynthetic rate) and C_i (substomatal CO_2 concentration). One of the main conclusions of those articles was that even though the decrease in g_s seemed to be responsible for the variation in water regulation status, the increase in C_i induced by Cr accumulation clears g_s as the responsible for the decrease in *A*. This also indicates, as hinted by Vernay *et al.* (2007) and Subrahmanyam (2008) that the reduction in *A* might lay in the functional status of the Calvin cycle enzymes. Unfortunately, the availability of data regarding Cr's putative effects on the enzymes of the Calvin cycle is far less than what exists for other parameters. The recent works of Dhir *et al.,* (2009) and Bah *et al.,* (2010) provided some of the first insights to Cr-induced effects at the Calvin cycle enzymes. Dhir *et al.,* (2009) found a significant decrease in RuBisCO activity induced by exposure to wastewaters (rich in Cr) from an electroplating unit and suggested that this results could be explained by: a substitution of Mg^{2+} in the active site of RuBisCO subunits by metal ions; decline in RuBisCO content as a result of oxidative damage and by a shift in the enzyme's activity from carboxilation to oxygenation. On the other hand, Bah *et al.,* (2010) performed a proteomic analysis of *Typha angustifolia*'s leaves exposed to metals and found that exposure to Cr induced the expression of ATP synthase, RuBisCO small subunit and coproporphyrinogen III oxidase. They explained that their data were evidence of a protective mechanism against metal toxicity at the photosynthetic level, which might be responsible for the metal tolerance displayed by *T. angustifolia*. Furthermore, the same authors also suggested that the increased expression of ATP synthase was indicative of the high energetic requirements needed to cope with metal toxicity.

Another unexplored endpoint of Cr-induced stress at the metabolic level is the variation in the amount of soluble sugars and starch accumulated in leaves. Besides being the fuel for carbon and energy metabolism, sugars also play a pivotal role as signaling molecules (Rolland *et al.,* 2006). Therefore, the quantification of the sugar levels in the leaves could provide information of paramount importance in the characterization and understanding of Cr-induced phytotoxicity.

Despite the appalling lack of data, reports like the ones presented by Tiwari *et al.,* (2009) and Prado *et al.,* (2010) offer some light into the effects of Cr at this level. Tiwari and co-workers found that exposure to increasing concentration of Cr caused a decrease in the amount of non-reducing sugars while the inverse was observed for reducing sugars. Prado *et al.,* (2010) on the other hand observed that Cr exposure caused the levels of sucrose (transport sugar) to increase while the concentration of glucose decreased.

Genotoxicity

In animals and yeast, Cr (VI) has been extensively studied and shown to be highly toxic, inducing cell cycle arrest and causing carcinogenic effects (*e.g.* Zhang *et al.,* 2001, O'Brien *et al.,* 2002, Salnikow and Zhitkovich, 2007). Despite of the critical importance of Cr toxicity, we are still far away of having in plants, the same level of understanding that exists in other eukaryotes about the mechanism and effects of Cr genotoxicity.

What serves as bases for understanding Cr genotoxicity in plants is what is known in other organisms; Cr is a special case among metals because unlike most of the other elements; once inside the cell, it interacts primarily and directly with DNA, forming DNA-protein and DNA-DNA cross links, making this element a highly mutagenic and carcinogenic toxicant. By this, while most metals are considered weakly mutagenic, mostly acting indirectly through the inhibition of DNA repair machinery or by inducing ROS formation, Cr acts on DNA causing genotoxicity directly. Cr can also form complexes which can react with hydrogen peroxide and generate significant amounts of hydroxyl radicals that may directly trigger DNA alterations and other effects (Salnikow and Zhitkovich, 2007). The mechanism of Cr(VI) detoxification by reductases creates unstable forms of Cr that are known to create ROS, which are one of the most common causes of DNA degradation. It have been shown that Cr(V) reacted with isolated DNA to produce 8-hydroxydeoxyguanosine, whereas Cr(VI) performed this reaction only in the presence of the reductant glutathione (Faux *et al*., 1992). In cultured mammalian cells, Cr(VI) induced superoxide and nitric oxide production (Hassoun and Stohs 1995), whereas treatment of cells with Cr(VI) in the presence of glutathione reductase generated hydroxyl radicals. This ROS, besides degrading DNA, can also affect Mitogenic-Activated Protein Kinsases (MAPK), which cause the deregulation of cell proliferation (tumor inducing effect), causing mutagenicity through an indirect path, besides the aforementioned direct interaction with DNA (Beyersmann and Hartwig, 2008).

Most of what it's known about Cr genotoxicity in plants is summarized in table 1; for the most part, these investigations focused on Cr induced chromosomal aberration and micronuclei formation. This is not uncommon as both markers are fairly easy to apply and low tech, being routinely used as indicators of genotoxicity in standard ecotoxicological assay. Unfortunately, the level of information that can be taken from these markers is insufficient to characterize and understand the complexity of Cr genotoxicity. Nonetheless, there is also evidence of Cr related DNA degradation (Comet assay) and point-mutation (AFLP) and therefore more research would

definitively help to clarify the mechanism of Cr genotoxicity in plants, perhaps confirming that, at least at some extent, what is known for other organisms can also be applied to plants.

Table 1 Literature survey of Cr genotoxic effect in plants.

Species	Reference	Dose	Technique	Effects
V. faba	(Chandra *et al.*, 2004)	Tannery solid waste	Cytogenetic	Chromosomal and mitotic aberration
B. napus	(Labra *et al.*, 2004)	$K_2Cr_2O_7$ (10 to 200 mg l^{-1})	AFLP, SAMPL, DNA methylation analysis	Methylation changes, Mutation
A. thaliana	(Labra *et al.*, 2003)	$K_2Cr_2O_7$ (2, 4 and 6 mg l^{-1})	AFLP	DNA mutation
C. sativa	(Citterio *et al.*, 2003)	$K_2Cr_2O_7$ (25µg g^{-1} and 50 µg g^{-1} soil)	FCM	ND
T. repens	(Citterio *et al.*, 2002)	Contaminated soils from a steelworks- Up to 4810 mg kg^{-1} soil	AFLP, FCM	Mutation, DNA decrease
V. faba	(Wang, 1999)	Cr (contaminated soils)	MN	Dose-related increase of MN
A. cepa	(Matsumoto *et al.*, 2006)	Tannery effluent	Cytogenetic	Chromossomal aberration
V. faba	(Koppen and Verschaeve, 1996)	$K_2Cr_2O_7$ (Up to 10^{-3}M)	Comet assay	Increase in %Tail DNA, Tail moment and Tail length
Tradescancia sp. ***V. Faba***	(Knasmuller *et al.*, 1998)	$CrCl_3$, CrO_3 (from 0.75 to 10 mM)	MN	Dose-related increase of MN for *Tradescancia*, ND in *V. faba*

Lead: the element

Lead (Pb) is a silvery-white highly malleable metal, with a low melting point and high density. Pb have had many applications since its discovery: The Egyptians used grounded lead ore as eyeliner with therapeutic proprieties; Pb based pigments were used as part of yellow red and white paint; in ancient Rome Pb was used to build pipes for water transportation and not so long ago, tetraethyl lead was used in petrol fuels. Nowadays, this metal is a major constituent of the batteries used in automobiles, in projectiles for firearms and molten Pb is used as a coolant.

Releases of lead in the environment can occur naturally from the mobilization of Pb from the Earth's crust and mantle, such as volcanic activity and the weathering of rocks. However, these releases are very rare and the most significant sources of Pb discharge are those originated by anthropogenic activities (United Nation Environmental Program, 2010).

Some of the most influential sources of Pb pollution are lead impurities in raw materials such as fossil fuels and other extracted and treated metals, mining, releases from incineration and installations for municipal waste, open burning and the mobilization of historical Pb releases previously deposited in soils, sediments and wastes. From the sources, this pollutant can be

transported thousands of kilometres through the air (burned fuel and air-borne particles like fly ash), by rivers and oceans (discharges from industries and leakage from residues) or by humans transportation.

In the atmosphere, lead will deposit on surfaces or exist as a component of atmospheric particles as lead compounds, being that the residence time range from hours to weeks. In the aquatic environment, lead can occur in ionic form (highly mobile and bio-available), organic complexes with dissolved humus materials (binding is rather strong and limits availability), attached to colloidal particles such as iron oxide (strongly bound and less mobile when available in this form than as free ions) or to solid particles of clay or dead remains of organisms (very limited mobility and availability) (United Nation Environmental Program, 2010).

Lead: Uptake and assimilation by plants

The speciation of lead differs whether it is in fresh water, seawater or soil. In fresh water lead primarily exists as the divalent cation (Pb^{2+}) under acidic conditions; under alkaline conditions it preferentially forms $PbCO_3$ and $Pb(OH)_2$. Lead speciation in seawater is a function of chloride's concentration and the primary species are $PbCl_3^-$ > $PbCO_3$ > $PbCl_2$ > $PbCl^+$ > and $Pb(OH)^+$. In soil, lead is generally not very mobile. The downward movement of elemental lead and inorganic lead compounds from soil to groundwater by leaching is very slow under most natural conditions. Clays, silts, iron and manganese oxides, and soil organic matter can bind lead and other metals electrostatically (cation exchange) as well as chemically (specific adsorption). Biotic factors like soil pH, content of humic acids and amount of organic matter influence the content and mobility of lead in soils. Despite the fact that lead is not very mobile in soil, lead may enter surface waters as a result of erosion of lead-containing soil particles. All these factors will influence the bioavailability of Pb and thus the toxicity level of this heavy metal (United Nation Environmental Program, 2010).

To become metabolized by plants, elements need to be transported, at some point, through the plasma membrane of the roots. Kučera *et al.,* (2008) reported that once in contact with plants, Pb was transported by CPx-type ATPases, a subgroup of P-type ATPases, that pump essential and non-essential metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} across the plasma membrane (Figure 2)

Plants absorb Pb usually accumulating it in the roots (Carruyo *et al.,* 2008, Hanc *et al.,* 2009), acting like a natural barrier. A small portion can also be translocated upwards to stems, leaves (Hanc *et al.,* 2009) and probably seeds; the increase in accumulation level is directly proportional to the amount of exogenous lead.

Authors have studied the effect of pH variation in Pb uptake, in different plant species: in low pH soils (3.9) an increased mobility of lead was observed, resulting in higher uptake (Ernst *et al.,* 2000). Gorlach *et al.,* (1990), working with Italian ryegrass found that with increasing soil pH (3.9–6.7) Pb uptake was reduced. Also, and in addition to soil factors, the species and genotype also play a role in Pb's uptake and accumulation.

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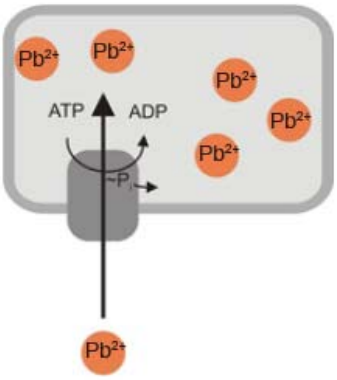

Figure 2 Pb transport by CPx-type ATPases (adapted from the model of Dr. Mathias Lübben)

Once inside the root cortex, Pb moves in the apoplastic space, using the transpiration conductive system (Wierzbicka, 1999, Hanc *et al.,* 2009). It can also bypass the endodermis and gain symplastic access in the young root zone and in sites of lateral root initiation (Eun *et al.,* 2000). Pb has been shown to enter and move within the cytoplasm and proteins mediating cross-membrane movement of Pb have been identified (Kerper and Hinkle, 1997, Arazi *et al.,* 1999). Most of the Pb absorbed by roots is in the form of extracellular precipitate (as phosphate and carbonate) or is bound to ion exchangeable sites in the cell walls (Sahi *et al.,* 2002). The unbound Pb is moved through Ca channels accumulating near the endodermis (Huang and Cunningham, 1996, Antosiewicz, 2005). Depending of the plants exposed, different cellular types can be used to store Pb: In wheat, Pb is fixed to the cell wall of roots but it can be removed as a complex using citric acid (Varga *et al.,* 1997). On the other hand, Peralta-Videa *et al.* (2009) discuss the accumulation of Pb in the Phloem tissues of *Prosopis* sp. associated with *Glomus deserticola,* suggesting that it was transported to the leaves and returned through the phloem to the plant organs.

Lead: Phytotoxicity

General effect

Metals induce several cellular stress responses and damage to different components such as membranes, proteins and DNA (Waisberg *et al.,* 2003). The first reported uses of lead date back to 4000 BC, and toxicological effects have been linked to lead since antiquity. Lead can be bioaccumulated by most organisms, whereas it is generally not biomagnified up the food web (United Nations Environment Program, 2010)

Pb its known to negatively affect some of the most classical endpoints of plant toxicity like germination rate, growth and dry mass of roots and shoots (Munzuroglu and Geckil, 2002; Ekmekçi *et al.,* 2009). In general, effects are more pronounced at higher concentrations and durations. In some cases, lower concentrations can stimulate metabolic processes and the enzymes involved in

those processes. The major endpoints are seed germination, seedling growth (shoot and root growth), photosynthesis, plant water status, mineral nutrition, and enzymatic activities. Visible symptoms include chlorotic spots, necrotic lesions in leaf surface, senescence of the leaf and stunted growth. Germination of seeds is drastically affected at higher concentrations and the development and growth of root and shoot in seedling stage is also affected, with roots being more sensitive (Li *et al*., 2005).

Lead reduces the uptake and transport of nutrients in plants, such as Ca, Fe, Mg, Mn, P and Zn, by blocking the entry or binding of the ions to ion-carriers making them unavailable for uptake and transport from roots to leaves (Xiong, 1997). This in turn interferes with several physiological and biochemical processes, among which photosynthesis is one of the most affected.

Photosynthesis

Photosynthesis is one of the most sensitive processes to lead: The substitution of the central atom of chlorophyll (Mg) by lead prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a breakdown of photosynthesis (Küpper *et al.,* 1996). Higher concentrations of lead significantly affected plant water status causing water deficit.

The deleterious effects of this metal in several physiological parameters have been addressed in several species: John *et al.,* (2009) found in *Brassica juncea* exposed to this metal, growth impairment and decrease in pigments content; Kosobrukhov *et al.* (2004) working with *Platango major* showed that Pb can affect the g_s, pigment content, and light and dark reactions; Bibi and Hussain (2005) demonstrated that the *A*, *E* and g_s of *Vigna mungo* plants were significantly affected when exposed to Pb. The total chlorophyll content and relative content proportion of Chl *a* and *b* were reduced, through inhibition of chlorophyll biosynthesis (Van Assche and Clijsters, 1990, Sengar and Pandey, 1996, Ernst *et al.,* 2000). Cenkci *et al.,* (2010) found that the content in carotenoids was less affected than chlorophylls by Pb and suggested that this was so because carotenoids protect chlorophyll from photo-oxidative destruction and thus, a reduction in carotenoids could have a serious consequence on chlorophyll pigments. Limitation of photosynthesis by reduced activity of Calvin cycle enzymes, *e.g.* RuBisCO activity, was reported for several plant species exposed to Pb (Vojtěchová and Leblová, 1991, Moustakas *et al*., 1994). Lee and Roh (2003) found that exposure to Cd induced significant decrease in RuBisCO activity which was associated to the amount of RuBisCO protein; this might be a hint to the decrease in RuBisCO activity observed with Pb exposure, it is possible that Pb as Cd cause a decrease in RuBisCO protein. Recently, Bah *et al.,* (2010) proved that Pb caused the up-regulation of carbohydrate metabolic pathway enzymes; APX and GRSF; RuBisCO activase, Mg-protoporphyrin IX chelatase, fructokinase, a chloroplast precursor and plastocyanin. The authors state that this was indicative of a strategy to cope with Pb toxicity, by increasing carbohydrate metabolism (fruktokinase), photosynthesis (RuBisCO activase, Mg-protoporphyrin chelatase and plastocyanin)

and defense response (APX and GRSF). They also concluded that despite that the strategy was responsible for the high tolerance of *T. angustifolia* to Pb toxicity this had a high energetic cost.

Transpiration intensity, osmotic pressure of cell sap, water potential of xylem, and relative water content were significantly reduced after 24 and 48h of exposure to Pb (Parys *et al.,* 1998). Lead also reduces the size of stomata but increases their number and diffusion resistance.

The mechanism(s) of metal toxicity on photosynthesis is still a matter of speculations (*e.g*. Figure 3), this may be partly due to the differences in experimental design, but it almost certainly involves electron transport in light reactions and enzyme activity in the dark reactions (Romanowska *et al.,* 2006). Despite of the fact that the mechanism by which Pb affects the photosynthetic apparatus is unclear, evidence indicates that this metal causes severe effect to the photosynthetic status of plants and thus, it is of vital importance to carry studies to better understand Pb's toxicity.

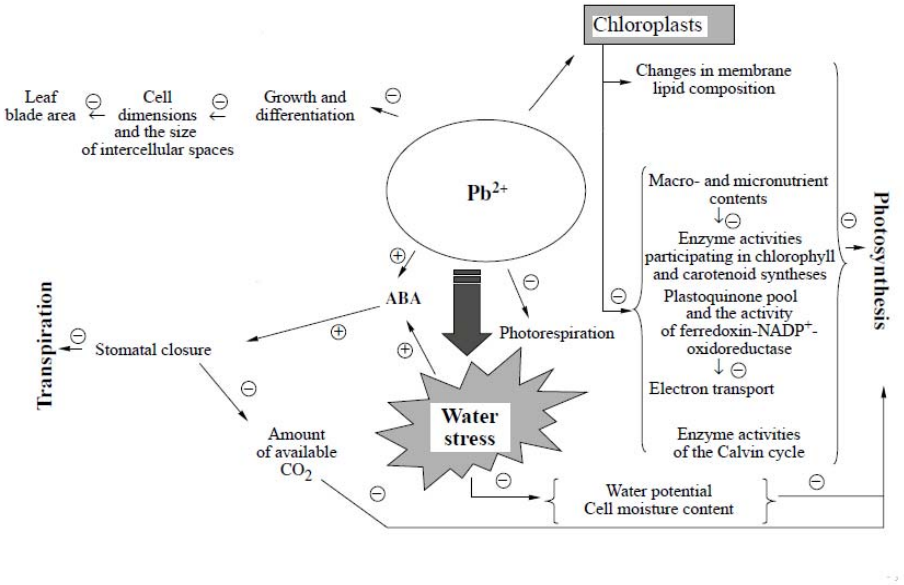

Figure 3 Effect of Pb on photosynthesis, respiration, and water regime. – Indicates decrease/inhibition and + indicates increase/activation. Taken from Seregin and Ivanov, (2001).

In Chapter III-2 and III-3, Pb and Cr -induced toxicity on the photosynthetic status of *Pisum sativum* plants, an important crop species both as human's food and as fodder for livestock, is addressed. The endpoints measured evaluated gas exchange, Calvin cycle enzymes activity, amount of soluble sugars and starch, pigment content and fluorescence emission. Moreover, chloroplast structure and functional status of these plants were evaluated by flow cytometry (FCM), in what it is an innovative approach of this technique to photosynthetic studies. In fact, in order to apply FCM to study heavy metal effect in chloroplast status, it was necessary to evaluate the suitability of this technique for this type of assays. To this end, a preliminary investigation was undertook and it is presented in chapter III-1: FCM data was correlated with PAM fluorometry and

pigment content, in a study in which pea plants were exposed to a classical known inhibitor of PSII electron transporter chain, Paraquat.

Genotoxicity

The chemical form of Pb only affects lead transport from the medium into the plants and all forms had similar effects on mitosis. The iodides had a greater mutagenic effect than the nitrates, perhaps because the latter dissolves completely in solution and are supplied as ions, rather than molecules as in the cases of the iodides (Radecki *et al.,* 1989).

Pb toxicity has been linked to carcinogenicity and the genotoxic effects of this metal have been studied thoroughly in animals and humans. Nevertheless, data related to the mutagenic, clastogenic and carcinogenic properties of inorganic lead compounds are still conflicting (García-Lestón *et al.,* 2010). Hartwig et al. (1990) working with V79 Chinese hamster cells exposed to Pb and UV radiation concluded that Pb alone did not induced DNA damage but magnified that caused by UV rays, this they said, was due to Pb interference with the repair machinery. This might be due to Pb ability to substitute calcium and/or zinc in enzymes involved in DNA processing and repair leading to an inhibition of DNA repair and an enhancement in the genotoxicity when combined with other DNA damaging agents (García-Lestón *et al.,* 2010). Valverde et al. (2001) demonstrated that despite that Pb did not cause DNA damage in acellular DNA, production of lipid peroxidation and an increase in free radical levels was observed, suggesting that Pb exposure could cause genotoxicity and carcinogenicity by indirect interactions, such as oxidative stress. These investigations support the current thesis (Figure 4) stating that the mechanism of action of Pb induced genotoxicity is originated by ROS formation and interference with the DNA repair mechanism (Beyersmann and Hartwig, 2008), rather than a direct interaction with DNA as it's seen with Cr.

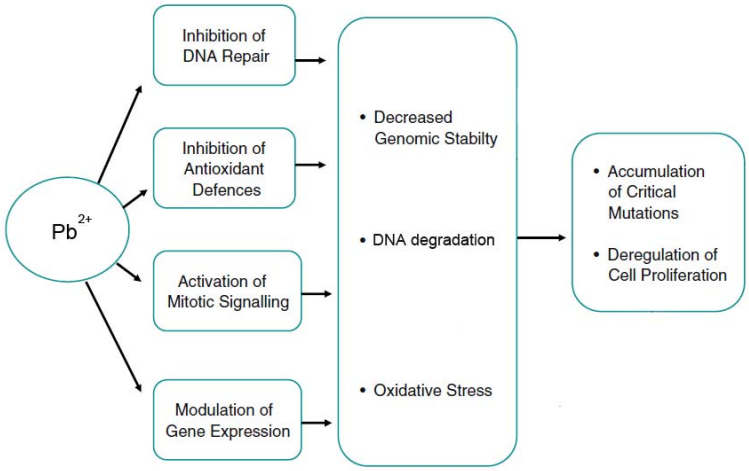

Figure 4 Major mechanism of Pb genotoxicity. Adapted from Beyersmann and Hartwig (2008).

Cell proliferation has also been demonstrated to be affected by Pb exposure, by increasing proliferative lesions in the kidney, below cytotoxic concentrations. This indicates that genotoxicity and accelerated growth stimuli may act in concert in lead-induced carcinogenicity (Beyersmann and Hartwig, 2008).

In plants and despite of the importance of Pb pollution and risk associated to the environment, the mechanism and effects of Pb toxicity are far less known than in animals (table 2 for an overview). Most of the Pb absorbed remains in the roots with only a small fraction being translocated to the shoots (Patra *et al.,* 2004). There, besides the ubiquitous chromosome aberration and micronuclei formation assays (*e.g*. Carruyo *et al.,* 2008, Shahid *et al*., 2011); Pb has been shown to cause DNA degradation in lupin and tobacco (Rucinska *et al.,* 2004, Gichner *et al.,* 2008b) by Comet assay and genomic instability (RAPD) in turnips (Cenkci *et al.,* 2010). Lead nitrate proved to be a weak mutagen but owing to its high toxicity had a synergistic effect in combination with ionizing radiation in some populations (Patra *et al.,* 2004).

Table 2 Literature survey of Pb genotoxic effect in plants.

Species	Reference	Dose	Technique	Effects
P. sativum	(Gabara *et al.,* 1995)	10^{-4} M	DNA synthesis	Diminished DNA synthesis
V. faba	(Chang-qun and Huan-xiao, 1995)	Pb^{2+} (NR)	cytogenetic	Mitotic stage shortened and interphase prolonged
A. cepa	(Rank and Nielsen, 1998)	Wastewater Sludges	cytogenetic	Anaphase-Telophase chromosome aberration
H. vulgare ***C. sativum*** ***A. cepa***	(Bhowmik, 2000)	$Pb(NO_3)_2$ (0.001 to 1 mg^{-1} Kg)	cytogenetic	Redution of mitotic index, increase of chromosomal aberration, polyploidy
A. thaliana **(transgenic)**	(Kovalchuk and Yao, 2011)	Pb^{2+} (0.002 to 0.83 mg l^{-1})	Trasngenic plant reporter gene	Increase in the mutation frequency
L. luteus	(Rucinska *et al.,* 2004)	$Pb(NO_3)_2$ (150 and 350 mg l^{-1})	Comet assay	DNA damage
N. tabacum	(Gichner *et al.,* 2008b)	Pb^{2+} (200 µM to 0.4 mM)	Comet Assay	DNA damage
B. rapa	(Cenkci *et al.,* 2010)		RAPD	Genomic template instability
L. Sativa	(Ritambhara and Girjesh, 2010)	$Pb(NO_3)_2$ (25 to 300 ppm)	cytogenetic	Abnormal chromosome migration
V.faba	(Shahid *et al.,* 2011))	$Pb(NO_3)_2$ (5 µM)	cytogenetic	MN and mitotic index

Chapter II-1, II-2 and II-3 presents an evaluation of Pb and Cr genotoxicity, respectively, in *P. sativum* plants, using flow cytometry, Comet assay and microsatellite to evaluate DNA damage, cell cycle arrest and microsatellite instability (MSI). Flow cytometry (FCM) is a technique that can easily excel in genotoxic studies, allying high analytical speed with multiparametric analysis (with a single analysis can provide information on variations in DNA content and polyploidization, variations in cell cycle dynamics and also, DNA damage). In plants, FCM has been demonstrated to be capable of detecting differences in DNA content as small as 1% (Pfosser *et al.,* 1995), chromosome aberration in wheat-rye lines exposed to aluminium (Rayburn and Wetzel, 2002), DNA damage in lettuce plants exposed to Cd (Monteiro *et al.,* 2010) and cell cycle arrest in *A .cepa* exposed to X-ray radiation (Carballo *et al.,* 2006).

Another good technique for assessing genotoxicity is the Comet assay, which is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins *et al.,* 2008). The simplicity inherent to sample preparation and the relatively small number of cells/nuclei analysis required to obtained robust results (Hattab *et al.,* 2009a), the later which can be automated further reducing the time need to obtain results, can be accounted as the reasons for the dramatic increase of Comet assays application in genotoxicity studies. In plants, the Comet assay has been proved to be very useful to study genotoxicity of heavy metals (*e.g* Hattab *et al.,* 2009a, Gichner *et al.,* 2006, Gichner *et al.,* 2008a). Lead in particular, have been demonstrated to cause genotoxic effects at short term exposure, in tobacco (Gichner *et al.,* 2008b) and lupin (Rucinska *et al.,* 2004), thus demonstrating the suitability of this technique to detect DNA damage caused by Pb.

Microsatellites or simple sequence repeats (SSR) are DNA sequence with tandem repeats (2-6 bp long) which have random and wide distribution throughout eukaryotic genomes and present high polymorphism and mutation rates (Burstin *et al*., 2001). Commonly used as a molecular marker in genetics (*e.g*. mapping and population studies), SSRs can be used in genotoxicity studies to evaluate MSI, which is indicative of a deficient functioning of the DNA mismatch repair system. For these reasons, SSRs has been used as clinical marker in certain types of cancer (Ribic *et al*., 2003) and also in Cd induced genotoxicity in animals (El- Ghor *et al*.,2010) and plants (Monteiro *et al*., 2009), opening good perspectives to assess metal-induced genotoxicity.

Objectives

Metal toxicity is both and an environmental and human health concern that requires constant vigilance and precise and unbiased evaluation. Plants, as primary producers of the food chain and sources of many products are extremely important for society and due to their sessile nature, are one of the groups which are more affected by pollution, which eventually reflects in their final consumers (human or animals). In order to understand and evaluate heavy metal genotoxicity and cytotoxicity, thorough investigations must be carried on, applying sensitive, rapid and robust techniques to accesses the deleterious effects of these contaminants. There for, the main objective

of the studies comprising this thesis was to evaluate the genotoxic and cytotoxic effects of two very important heavy metal pollutants, Cr(VI) and Pb^{2+}, in one of the most important edible crops, *Pisum sativum*.

To fulfill the main objective the following questions were addressed:

i. Does Cr and Pb cause DNA degradation, DNA content variation and changes in cell cycle dynamics?

ii. What is the level of phytotoxicity of these heavy metals in photosynthesis and which endpoints are more sensitive to their deleterious effect?

iii. It's there any relationship between Cr and Pb toxicity?

To answer these questions the following tasks were performed

a) Evaluation of Pb genotoxicity and cytotoxicity in chapter II-1

b) Evaluation of Cr genotoxicity and cytotoxicity in chapter II-2 and II-3

c) Establishment of FCM as a suitable protocol to evaluate toxicity in photosynthesis, namely through the analysis of chloroplast structure and fluorescence status, in chapter III-1

d) Analysis of Pb's effect in the photosynthetic status using classical and cutting-edge endpoints, in chapter III-2

e) Analysis of Cr's effect in the photosynthetic status using classical and cutting-edge endpoints, in chapter III-3

In the final chapter of the dissertation, chapter IV, a global discussion about the chapters and the final remarks and conclusions of the investigation presented in this thesis, are presented.

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CHAPTER II

METAL-INDUCED GENOTOXICITY IN PLANTS

CHAPTER II-1

GENO AND CYTOTOXICITY IN *PISUM SATIVUM*: CELL CYCLE ARREST AND DNA DAMAGE AS CONSEQUENCE OF PB^{2+} EXPOSURE

Chapter submitted as an original article to an SCI journal

Rodriguez E, Azevedo R, Moreira H, Fernandes P, Souto L and Santos C (2011). Geno and cytotoxicity in *Pisum sativum*: cell cycle arrest and DNA damage as consequence of Pb^{2+} exposure. Metallomics. (submitted).

Abstract

Despite known for centuries as a toxic element and risk to human health, studies on Lead (Pb) effect and mechanisms of toxicity have produced contradictory data. In plants this scenario is aggravated by the lack of information on Pb phytotoxicity. In order to evaluate Pb cyto and genotoxicity, *Pisum sativum* L. plants were grown on soil and watered with different Pb^{2+} solutions with the following concentrations: 0, 20, 200, 1000 and 2000 mg l^{-1}. After 28 days, leaves showed no significant variations in any of the parameters analyzed, except for an increase in DNA degradation at the highest Pb^{2+} concentration. As for the roots, significant changes in cell cycle dynamics were observed at G_0/G_1 and G_2, being that the maximum dosage of Pb administrated induced an arrest at the G_2/M checkpoint. DNA damage analysis by Comet assay and flow cytometry revealed significant increases in tail moment (TM) and full peak coefficient of variation, respectively, for the highest concentrations. Moreover, the evaluation of microsatellite instability (MSI), which serves as an indicator of the status of the mismatch repair system (MMR), demonstrated that the highest dosage used caused changes in one microsatellite. In conclusion, we demonstrate that Pb^{2+} severely affects the parameters tested at the highest concentration analyzed. We hypothesize that Pb^{2+} induced genotoxicity (e.g. DNA damage and MSI) are responsible for the cell cycle arrest at the G_2/M checkpoint.

Keywords: cell cycle arrest; DNA degradation; genotoxicity; microsatellite instability; Pb; *Pisum sativum.*

Introduction

Metal pollution is a growing concern due to their high toxicity, easiness to spread and to the fact that these toxicants cannot be degraded, tending to persist in the environment for considerable periods of time. The start of the industrial revolution (18th and early 19th century) is often associated to the dramatic increase of metal concentration in the environment. Notwithstanding, historical accounts indicate that lead (Pb) pollution dates much earlier; the toxic effects of this metal (*e.g.* anemia and developmental mental disturbances) has been described in documents from the roman empire (Hernberg, 2000), being that the major sources of Pb release were smelters and mines (Nriagu, 1996). More recently, studies in vivo, animals and humans have linked this metal to genotoxicity and carcinogenicity (Beyersmann and Hartwig, 2008). Nevertheless, data related to the mutagenic, clastogenic and carcinogenic properties of inorganic lead compounds are still conflicting (García-Lestón *et al.,* 2010). It is currently believed that Pb^{2+} induces genotoxic effects by an indirect mechanism, through the generation of ROS and by interfering with the DNA repair mechanism (Beyersmann and Hartwig, 2008).

In plants and despite of the importance of Pb^{2+} pollution and the associated risk to the environment, the level of comprehension regarding the mechanism and effects of Pb^{2+} toxicity is inferior to what is known for other organism. The current model states that most of the Pb^{2+} absorbed remains in the roots with only a small fraction being translocated to the shoots (Patra *et al.,* 2004). Pb^{2+} has been shown to cause chromosome aberration in *A. cepa* (Carruyo *et al.,* 2008), DNA degradation (by Comet assay) in lupin and tobacco (Rucinska *et al.,* 2004, Gichner *et al.,* 2008c) and genomic instability in turnips (Cenkci *et al.,* 2010).

Due to its high toxicity, Lead pollution has become an important concern, having been deemed as a priority hazardous contaminant by EU (Annex II of the Directive 2008/105/EC). Therefore and for all of the exposed so far, It is highly relevant to perform a comprehensive and thorough evaluation of this metal genotoxic effects. To achieve this it is important to use sensitive and robust techniques, which can then be applied to assess genotoxicity endpoints and assist in elucidating the mechanism by which this pollutant induces genotoxicity.

Flow cytometry (FCM) is a technique that can easily excel in genotoxic studies, allying high analytical speed with multiparametric analysis (with a single analysis can provide information on variations in DNA content and polyploidization, variations in cell cycle dynamics and also, DNA damage). In plants, FCM has been demonstrated to detected differences in DNA content as small as 1% (Pfosser *et al.,* 1995), chromosome aberration in wheat-rye lines exposed to aluminium (Rayburn and Wetzel, 2002), DNA damage in lettuce plants exposed to Cd (Monteiro *et al.,* 2010) and cell cycle arrest in *A. cepa* exposed to X-ray radiation (Carballo *et al.,* 2006).

Another reliable technique for assessing genotoxicity is the Comet assay, which is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins *et al.,* 2008). The simplicity inherent to sample preparation and the relatively small number of cells/nuclei analysis required to obtained robust results (Hattab *et al.,* 2009), the later which can be automated further reducing the time need to obtain results, can be accounted as the reasons for

the dramatic increase of Comet assays application in genotoxicity studies. In plants, Comet assay has been proved to be very useful to study genotoxicity of heavy metals (*e.g* Hattab *et al.,* 2009, Gichner *et al.,* 2006, Gichner *et al.,* 2008b, Murali Achary and Panda, 2010). Despite that Pb^{2+} genotoxicity has been assessed by this technique in plants (Rucinska *et al.,* 2004, Gichner *et al.,* 2008c), the applicability of this technique to detect genotoxicity in plants grown on soil has been questioned and so, the usefulness of Comet assay to evaluate DNA damage in environmental conditions has yet to be proven.

With the development of PCR based techniques, the study of the mechanism involving metal-induced genotoxicity at the molecular level has become easier and more accessible. Among the many molecular markers which can be used, microsatellites (or simple sequence repeats, SSR) are among the most informative and robust, presenting high mutation rates (Sung *et al.,* 2010). SSR are tandem repeats of DNA sequences of 2–6 base pair (bp) long, which are highly abundant, have random distribution through eukaryote genomes and high degree of polymorphism (El-Ghor *et al.,* 2010). Moreover, Microsatellite instability (MSI), manifested as repeat length polymorphisms, is a hallmark of mismatch repair (MMR) system deficiency, being used in clinical trials as a marker for cancer (Leonard *et al.,* 2003) but also with applications in animals (El-Ghor *et al.,* 2010) and plants (Leonard *et al.,* 2003, Monteiro *et al.,* 2009, Golubov *et al.,* 2010). Being that metal induced genotoxicity is currtenly thought to affect the DNA repair machinary (García-Lestón *et al.,* 2010) it is important to clarify if Pb can induce this type of problem in plants.

In the investigation presented here, we analyzed the genotoxic effect of Pb^{2+} in the model crop species *Pisum sativum*. To that effect, plants grown in soil were exposed, for 28 days to in $PbCl_2$ solution (Pb^{2+}) with concentrations ranging 20 mg l^{-1} (maximum admitted level in some European Union countries for agricultural purpose waters) to 2000 mg l^{-1}. The following endpoints were evaluated: Cell cycle dynamics, ploidy level, DNA damage and MSI, in roots and shoots of these plants. Our data gives a new perspective on Pb^{2+} genotoxicity in plants giving a better understanding on lead's mechanism for generating toxicity. We also demonstrated the suitability and efficiency of FCM, Comet assay and SSR to evaluate genotoxicity endpoints.

Material and Methods

Plant material

Pea seeds (*Pisum sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sawed in peat:perlite mixture. Plants were grown during 28 days at 24ºC ± 1ºC, under light intensity of 200 μmol m^{-2} s^{-1}, photoperiod of 16/8 h (light/dark). Pb treatment consisted in watering plants (at least 25 per condition) during the aforementioned period of 28 days, twice per week, with 100 ml of a 1:10 Hoagland's solution containing the following $PbCl_2$ (referred to as Pb^{2+}concentration) concentrations: 0; 20; 200; 1000 and 2000 mg l^{-1}. Afterwards plants were collected, rinsed thoroughly and tissue sampled for analysis. At least two independent experiments were performed for each parameter.

Lead analysis

Pb^{2+} concentration in control and Pb^{2+}-treated plants (n=3) was verified by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France). Accumulation of Pb^{2+} was determined in leaves and roots dried to constant weight at 60ºC. Prior to drying, roots were washed for 10 min in 0.5mM $CaSO_4$ to remove (by cation exchange) Pb^{2+} adsorbed to the root surface. Dried tissues were treated according to Rodriguez *et al.,* (2011) and then analyzed by ICP-AES. The real Pb concentration in the growing media with nominal concentrations of, respectively, 0; 20; 200; 1000 and 2000 mg l^{-1} Pb, was also measured by ICP-AES.

Flow cytometry

For flow cytometry (FCM) analyses root tips (2 mm) and leaves were treated as follows: Tissue was placed on a Petri dish and chopped in 1 ml of woody plant buffer (WPB- Loureiro *et al.,* 2007), with a razor blade. Nuclei suspension was collected and filtered through a 55 µm nylon mesh to remove large debris. Afterwards, 50 µg ml^{-1} of propidium iodide (PI) and 50 µg ml^{-1} of RNase were added to label DNA and degrade RNA respectively- After 5 min incubation on ice, samples were analyzed in a Coulter Epics flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. The results were acquired using the SYSTEM II software version 3_0 (Coulter Electronics). Prior to analysis, the instrument was checked for linearity with fluorescent beads (Coulter Electronics), and the amplification settings were kept constant throughout the experiment.

The proportion of cells in each of the cell cycle phases, the full peak coefficient of variation (FCPV) of the G_0/G_1 peak and the CV of the G_0/G_1 were collected from the fluorescence intensity (FL) histograms (5 replicates per condition). For cell cycle analysis, flow cytometric files were transformed to single histograms files with WinMDI ver 2.8 software (Freeware by Joe Trotter of the Scripps Institute, La Jolla, CA.) and then analyzed using Cylchred software (Freeware developed by Terry Hoy of the University of Cardiff). Resultant data were treated using Microsoft Office Excel 2007.

For nuclear DNA content determination (10 plants per condition), *Vicia faba* cv Inovec, containing a DNA content of 26.90 pg per 2C, was used as an internal standard in order to determine the DNA content of *P. sativum* plants, as described by Loureiro *et al.,* (2007). At least 5000 nuclei were analyzed in each sample.

Comet assay

Roots and leaves were processed as described by Gichner *et al.,* (2008a), for the alkaline Comet assay procedure, with some modifications. Also, and to avoid light-induced DNA strand brakes, all the procedures were conducted under dim light. Samples were placed in a Petri dish kept on ice altogether with 20 ml ice-cold Tris buffer (400 mM, pH 7.5) and gently sliced with a razor blade. Frosted edge microscope slide were covered with 1 % normal melting point agarose (NMP) at 50ºC, dried over night and kept covered, in the dark, until usage. The nuclei suspension was placed in the slide together with 1% low melting point agarose (LMP) prepared in PBS (Phosphate Buffered Saline), mixed by gently pippeting, followed by covering the mixture with a cover slip and incubation on ice for 5 min. After this, the cover slip was removed, a layer of 0.5% LMP was added and a new cover slip was placed on the slide, which was incubated for 5 min on ice.

A parallel test of the unwinding and electrophoresis time was assessed, and the optimal times for *P. sativum* were chosen (data not shown). Nuclei were unwind by placing the slides in alkaline electrophoresis buffer for 15 min prior to electrophoresis at 0.74 V cm^{-1} for 15min. Afterwards, the slides were rinsed three times with 400 mM Tris buffer (pH 7.5), air-dried and stored until further use.

For analysis, the slides were hydrated in distilled water for 10min, stained with 80 µl ethidium bromide (20 µg ml^{-1}) for 5 min and excess stain was removed by rising in distilled water three times. The slides were then covered with a cover slip and analyzed with a Nikon Eclipse 80i with an excitation filter of 510-560 nm and a barrier filter of 590 nm.

Comet images were analyzed using CASP v1.2.2 software. At least three slides were evaluated per treatment (from three different individuals) and at least 25 nuclei per slide were exanimate. Values presented are given as the average of medians ± SE of at least 75 nuclei per condition. The % Tail DNA (TD) and the Tail moment (TM) values were recorded and compared.

Microsatellite analysis

Total DNA was extracted from roots and leaves (100 mg each) of control and Pb-treated plants (3 individuals per condition from 2 independent experiments), using the DNeasy® Plant Mini Kit (QIAGEN, Germany) as instructed by the manufacturer.

Ten SSR were chosen from the ones reported by Burstin *et al.,* (2001), to represent different repetitive motifs, size; locations in the sequence and number of repeats. The primers were synthesized by Invitrogen (UK). From the ten SSR chosen, 2 could not be amplified, even if the experimental conditions were changed and thus, the remaining 8 SSR (which amplified) were used to evaluate Pb-induced MSI. The primers, repetitive motif and annealing temperatures are given in Table 1. In order to choose the proper PCR conditions a pre-assay was performed, with the annealing temperatures ranging from 63 to 54 ºC. The optimal temperatures for each SSR are given in table 1. The remaining PCR conditions were as instructed by the PCR's kit manufacturer (TAQ PCR core Kit, Qiagen): each assay contained 30 ng of template DNA, in 20 pM of each

primer, 0.2 mM dNTP, 1*x* Taq buffer containing 1.5 mM $MgCl_2$, 1 U Taq polymerase in a total volume of 25 µl. PCR was performed in a MyiQ2 (Biorad, CA, USA) and included 1 step of 3 min at 94 ºC followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at the required Tm (Table 1) and 45 s elongation at 72°C. The final step was a 5 min elongation at 72°C.

Table 1 Description of the Microsatellite used. Locus, accession number, forward and reverse primers sequence and motif were taken from Burstin *et al*., (2001). Tm- temperature used. The size was calculated from the information in BLAST regarding each gene.

SSR	Locus	Accession Nº	Forward primer	Reverse primer	Motif	Tm (ºC)	Size (bp)
1	PEAATPSYND	M94558	CTCCAGCCCAATAGTCGAAG	TCACAACCGAAGTCACAACC	$(AC)_6$	61.5	208
2	PEACPLHPPS	L19651	GTGGCTGATCCTGTCAACAA	CAACAACCAAGAGCAAAGAAAA	$(AT)_6$	61.5	126
3	PSRBCS3C	X04334	CCCAGTGAAGAAGGTCAACA	CAATGGTGGCAAATAGGAAA	$(AT)_6$	57.5	210
4	PSARGDECA	Z37540	CTGTTCCTCTTTCAAGCACTCC	GGGAAAGCAAAGCATGCGGATC	$(TC)_7$	61.5	258
5	PEARHOGTPP	L19093	ACGCTTCAACGGCAAAAT	AGGACCCCAATCACTCTCAC	$(TC)_5$	57.5	195
6	PSGSR1	X04763	TGAAACCACCATTCTCTGGA	AAGACCCCACTTGAAAATTACTTC	$(ATT)_5$	55.9	196
7	PSAJ3318	AJ223318	CAGTGGTGACAGCAGGGCCAAG	CCTACATGGTGTACGTAGACAC	$(CAT)_6$	61.5	166
8	PSBT2AGEN	X96764	GCAGCAGAGCTTGTCTTTGAG	GGAATCAGAAACAGCCTTGGG	$(CCT)_5$	57.5	269

PCR products were electrophoresed in 2 % agarose gels stained with ethidium bromide and running in 1× TBE buffer. The bands were visualized on a UV transilluminator (G:Box, Syngene, Cambridge, UK). The respective software (GeneSnap) was used for image acquisition and analysis. PCR products were further evaluated by Capillary Electrophoresis (CE) on an ABI Prism 310 ® 310 Genetic Analyser (Applied Biosystems, USA). Each sample was prepared according to the modified protocol from Life Technologies Corporation: 1 µL of PCR product was mixed with 25 µl deionized formamide (HI-Di™ Formamide, Applied Biosystems). Also, 1.0 µL of internal size-standard labelled with ROX™ (GeneScan™ 500 ROX™ Size Standard, Applied Biosystems) was added to all the samples to be used as a reference with known size. To ensure that the results obtained were not due to artifacts, the analysis was performed in two independent experiments.

Statistical analysis

Statistical significance of treatments was assessed by One-Way ANOVA with a post-hoc Holm-Sidak multiple comparisons test, using SigmaStat 3.5 for WINDOWS (SPSS Inc., Chicago, IL, USA). Pearson's correlation among the endpoints tested was performed using SigmaPlot for Windows ver. 11.0 (Systat Software Inc).

Results

Overall characterization of plant growth and morphology

At a morphological level, Pb^{2+} toxicity was more evident at 1000 and 2000 mg l^{-1} dosages, being the effects more severe in roots than in leaves. Roots deterioration was visible starting at 200 mg l^{-1}, with roots becoming thinner, more fragile and acquiring a dark brown coloration, as the Pb^{2+} concentration increased. Leaves revealed signs of toxicity at 1000 mg l^{-1} but mainly at 2000 mg l^{-1}, presenting paler tones and frailer consistency in comparison to leaves from control plants.

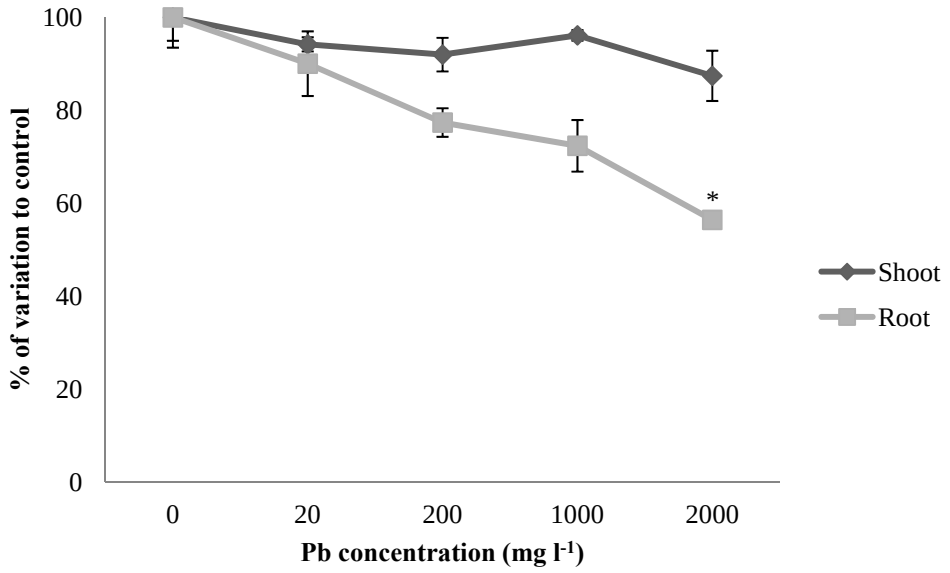

Figure 1 Length variation (%) of roots and shoots from plants exposed to the given concentration of Pb (mg l^{-1}), in respect to control tissues. Results are given as the mean ± SE of 5 replicates per condition. Values followed by (*) are significantly different from control ($P \leq 0.001$).

As for plant growth, Figure 1 presents the variation (in percentage) of roots and leaves exposed to Pb^{2+} in respect to control plants. Significant growth inhibition ($P \leq 0.05$) was observed in roots exposed to 2000 mg l^{-1} (decrease of 43.58 %) when compared to control roots. Shoots did

not show significant variations ($P > 0.05$) in size when compared to control plants (maximum decrease of 12.56% at 2000 mg l^{-1}).

Lead content in roots and shoots

The Pb concentration in the control (0 mg l^{-1}) medium was below the ICP-AES detection limit. In other growing media with Pb nominal concentrations of 20; 200; 1000 and 2000 mg l^{-1}, the measured values were, respectively, of 20.07 ± 0.004 mg l^{-1}; 200.09 ± 0.020 mg l^{-1}; 1000.13 ± 0.055 mg l^{-1}; 2000.26 ± 0.95 mg l^{-1} of total Pb.

The amount of total lead accumulated in roots and leaves of plants exposed for 28 days to this metal is presented in Figure 2. Tissues from exposed plants significantly accumulated more Pb^{2+} than control tissues ($P \leq 0.05$). Roots accumulated, at the lowest concentration, 11 fold more Pb^{2+} than leaves. While the highest difference in content between tissues was observed at 1000 mg l^{-1} (37 fold higher in roots), maximum Pb accumulation was detected in plants exposed 2000 mg l^{-1} with shoots having 109.9 ± 7.69 µg Pb g^{-1} DW and roots 3472.2 ± 255.48 µg Pb g^{-1} DW.

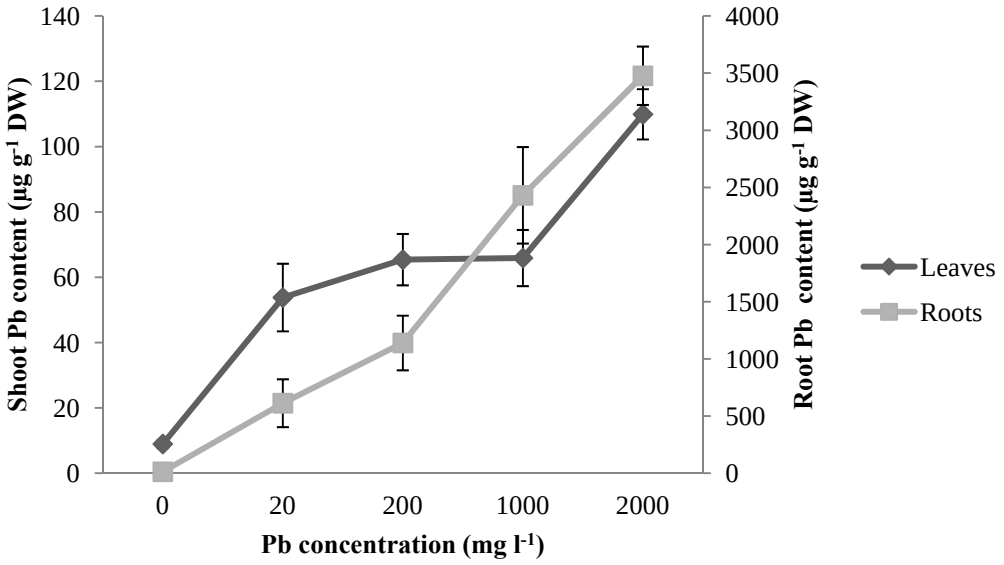

Figure 2 Lead content in roots and leaves exposed to the given concentration (mg l^{-1}). Results are displayed as the mean ± SE of 3 individuals per condition (run in triplicates). All values are significantly different from control ($P \leq 0.001$).

Flow cytometry

The flow cytometric analysis of nuclei extracted from both roots and leaves showed no statistical variation in either volume or granularity ($P > 0.05$), independently of the concentration tested (data not shown).

The FL histogram for control plants displayed a main peak, corresponding to nuclei at G_0/G_1 (84 % and 45 % of the events, for leaves and roots respectively) and a smaller peak corresponding to G_2 (around 8 and 40 %, for leaves and roots respectively). Control plants presented a mean CV value of 2.30 ± 0.138 and 3.40 ± 0.249, for leaves and roots respectively while the highest values for exposed tissues were observed in roots exposed to 200 mg l^{-1}, with 3.16 ± 0.160 and leaves exposed to 20 mg l^{-1} with 3.16 ± 0.258 (table 2).

The results obtained from the analysis of the G_0/G_1 peak FPCV revealed that Pb^{2+} exposure induced a significant increase in this biomarker. Roots exposed to 1000 mg l^{-1} and 2000 mg l^{-1} presented significant increases ($P \leq 0.001$) of 1.25 and 1.33 fold in respect to control (table 2). Leaves exposed to lead presented significant increases at all the dosages tested, in respect to control ($P \leq 0.05$), ranging from 1.30 to 1.38 fold (1000 mg l^{-1} and 200 mg l^{-1} respectively, though not significantly different among Pb dosages $P > 0.05$).

Table 2. Coefficient of variation (HPCV) and full peak coefficient of variation (FPCV) of roots and leaves of plants exposed to Pb. Values are given as mean ± SD of 5 individuals. (*) significantly different from control ($P \leq 0.001$)

	Pb [mg l^{-1}]	HPCV	SD	FPCV	SD
Leaves	0	2.30	0.138	3.24	0.196
	20	3.29	0.653	4.36*	0.691
	200	3.22	0.756	4.48*	0.404
	1000	3.24	0.285	4.19*	0.448
	2000	3.23	0.184	4.55*	0.496

	Pb [mg l^{-1}]	HPCV	SD	FPCV	SD
Roots	0	3.44	0.249	4.39	0.253
	20	2.29	0.516	4.77	0.470
	200	3.16	0.340	4.57	0.602
	1000	3.14	0.761	5.47*	0.271
	2000	3.04	0.117	5.83*	0.157

DNA content variation and ploidy level changes were not observed in any of the conditions (tissue/Pb^{2+} concentration) tested. The variation in DNA content were below 5% of the 9.10 pg 2C presented by control plants ($P > 0.05$).

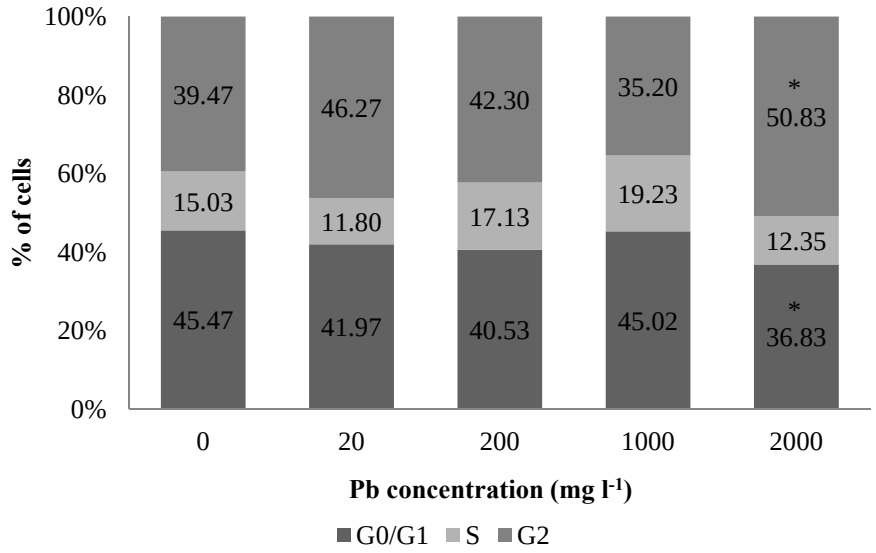

Figure 3 Cell cycle dynamics of roots exposed to Pb. Values are given as the mean % of cells in each of the cell cycle phases, of 5 individuals per condition. (*) significantly different from control ($P \leq 0.001$).

Cell cycle dynamics were also evaluated to assess putative cytostatic effects. In roots, exposure to 2000 mg l^{-1} caused a significant decrease of the proportion of cells in G_0/G_1 (20% in respect to control) and an arrest at G_2, with 50% of the cells analyzed being in this phase (Figure 3). This represents an increase of 22% in respect to control ($P \leq 0.05$). In leaves, the profile of cell cycle progression showed little variation among the dosages assayed ($P > 0.05$) (data not shown).

Comet assay

The TM and the TD were used as markers of the endpoint DNA damage in both roots and leaves of plants exposed to Pb^{2+}. Comparison between tissues, with either marker indicated that roots were more severely affected than leaves.

The analysis of the root's TM revealed that, except for the lowest concentration, all the dosages tested induced a significant increase in this marker ($P \leq 0.001$). There is a sharp increase in TM from 20 mg l^{-1} to 200 mg l^{-1} (nearly 8 fold); then, the maximum TM is observed at 1000 mg l^{-1} (433.2 ± 19.88), being significantly higher than all the other values ($P \leq 0.001$). At 2000 mg l^{-1}, the TM decreased to the level of damage observed at 200 mg l^{-1} ($P > 0.05$) (Figures 4 and 5).

Leaves of plants exposed to Pb^{2+} showed significantly higher values of TM ($P \leq 0.001$) than control (Figure 4). Like for roots, the highest TM value was detected with 1000 mg l^{-1} while at 2000 mg l^{-1} the level of damage was also similar to the one verified at 200 mg l^{-1} ($P > 0.05$).

Roots exposed to any of the Pb^{2+} dosages tested displayed a significant increase in TD, when compared to control roots ($P \leq 0.001$). Similar to what was observed for the TM, the maximum TD was observed in roots exposed to 1000 mg l^{-1} (70% of TD, 7 fold increase to control),

which was significantly higher than any other value observed ($P \leq 0.05$). Roots exposed to 2000 mg l^{-1} showed a decrease (Figure 4).to the same TD gamut observed at 200 mg l^{-1} ($P > 0.05$), like what was detected with the TM

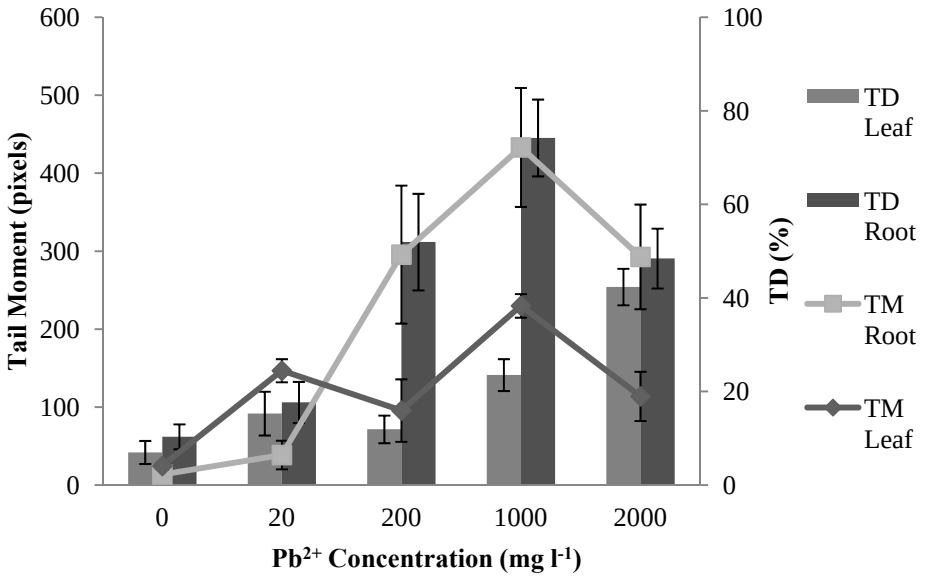

Figure 4 TD and TM of roots and leaves exposed to given Pb concentrations. Values are given as mean ± SD of at least 3 individuals, with at minimum of 25 nuclei per slide. TM values are given in arbitrary units (left axis). All values are significantly different ($P \leq 0.001$) from control except for the TM of roots exposed to 20 mg l^{-1} ($P > 0.05$).

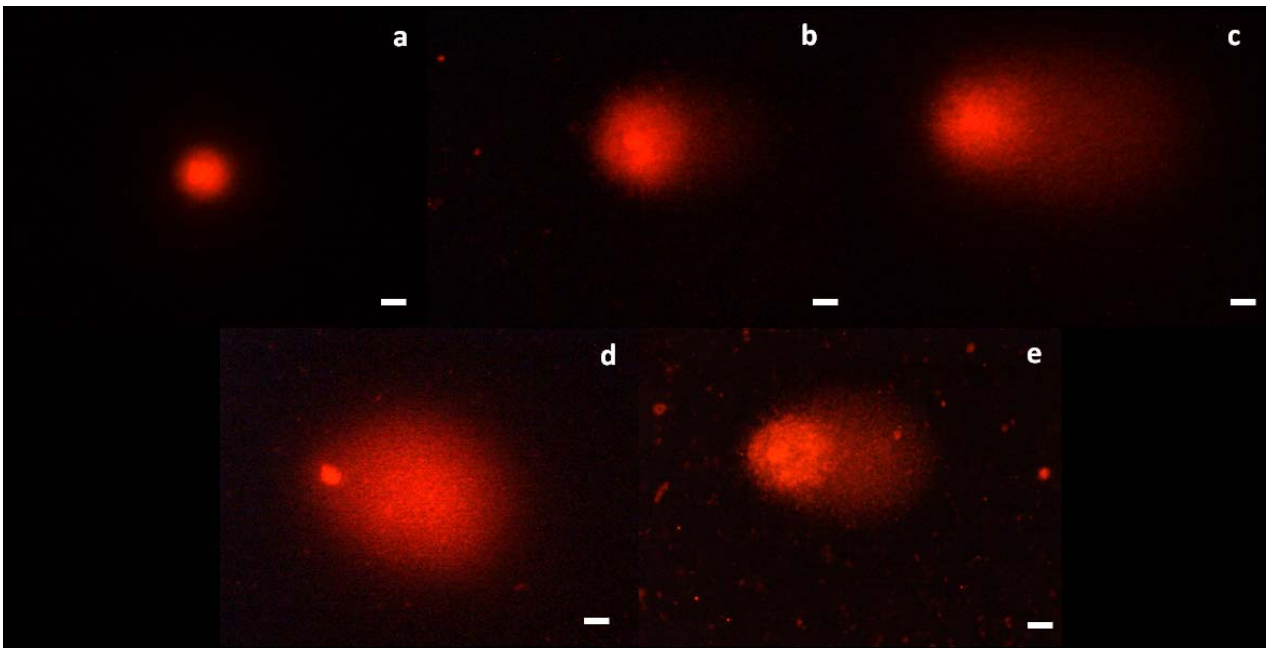

Figure 5 Comet assay representative images of nuclei extracted from roots exposed to the following concentration of Pb: a) 0 mg l^{-1}, b) 20 mg l^{-1}, c) 200 mg l^{-1}, d) 1000 mg l^{-1} and e) 2000 mg l^{-1}. Magnification scale bar (-) = 40×

Leaves of plants exposed to Pb^{2+} had significantly higher TD than control ($P \leq 0.001$), but unlike in all the aforementioned cases, the maximum value was observed in response to 2000 mg l^{-1} (42%), a 6 fold increase in respect to control leaves (Figure 4).

Microsatellite instability

The PCR fragment size of roots and leaves from control plants presented the same size and are displayed under the same column of table 3. It can also be observed that the predicted and the control's amplified fragment were of similar size except for SSR 5 which in our plants was 120 bp larger than the expected value.

Exposure to Pb did not induce significant variation in the fragment size of leaves, at any of the dosages used. As for roots, doses up to 1000 mg l^{-1} did not cause changes in the fragment's size but at 2000 mg l^{-1}, one of the individuals analyzed presented, for SSR6, a PCR product that was 3bp larger than that of the control (Table 3). The mutation frequency observed was of 4.2% (1 root plant/3 individuals) * (1SSR/8SSR analyzed).

Table 3. Allele size of the SSRs used to evaluate MSI. The values presented, in base pairs (bp), for each SSR are: the predicted size taken from the Blast database, the size obtained for control tissues, exposed leaves and exposed roots. In the case of Pb treated tissues, data for all doses tested are presented in the same columns because differences among treatments were not observed (with one exception).

SSR	Predicted Size (bp)	Control (bp)	Exposed Leaves (bp)	Exposed Roots (bp)
1	208	208	208	208
2	126	125	125	125
3	210	206	206	206
4	258	255	255	255
5	195	315	314	316
6	196	198	198	198; 201*
7	166	168	168	168
8	269	267	267	267

*One sample of a Pb-treated (2000 mg l^{-1}) root showed an allele of 201 bp

Discussion

Evaluating metal genotoxicity is of vital importance due to the recurring reports of contamination throughout the world and using sensitive and robust endpoints to correctly perform this task is an imperative condition.

The Comet assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins *et al.,* 2008). The most common DNA damage marker in plant applications (of the Comet assay) is the TM (Gichner *et al.,* 2008a, Gichner *et al.,* 2008c, Hattab *et al.,* 2009). However, recently, Collins et al (2008) suggested that despite this parameter may be more informative with small levels of damage, the TD covers the widest range of damage. Moreover the TD is linearly related to break frequency, allowing better inter-laboratory comparison (Collins *et al.,* 2008). To elucidate the suitability of these markers to evaluate Pb^{2+} induced DNA damage, we compared the TM and the TD. Despite that in a general way both gave similar results, the TD was more sensitive than the TM, detecting a significant increase in DNA damage, in roots exposed to 20 mg l^{-1} ($P \leq 0.05$), while the TM of roots exposed to this concentration was not significantly different from control ($P > 0.05$).

Another difference observed between both markers is that the highest TD in leaves was observed at 2000 mg l^{-1} while the maximum values observed for roots (TD and TM) and leaves (TM) was at 1000 mg l^{-1}, followed by a significant decrease ($P \leq 0.001$) at 2000 mg l^{-1} (to the level of damage observed at 200 mg l^{-1}).

In agreement with our data, Gichner *et al.,* (2008c), Rucinska *et al.,* (2004) and Wozniak and Blasiak (2003) working with tobacco, lupin (exposed to lead nitrate) and human lynphocytes (exposed to lead acetate) respectively, also found that the level of damage assessed by Comet Assay (by TM) decreased with exposure to high doses of Pb^{2+} (after 1.6 mM; 150 mg l^{-1}; and 100 µM, respectively), but they did not perform any comparisons between TD and TM. The most probable justification for this profile, as stated by those authors, is an inhibition of DNA migration during electrophoresis due to DNA–DNA and DNA–protein cross-links formation at high Pb^{2+} doses. It is interesting to note that the above mentioned pattern has been reported in such different conditions (*i.e*. species, doses and exposure time). For instance Gichner *et al.,* (2008c) short-termed exposure assay (24h to lead nitrate solution concentrations up to 2.4 mM) presented the maximum TM at 1.6 mM and steady decrease at higher doses, when tissues accumulated around 1368 and 51168 mg of Pb for kg of dry mass (shoots and roots respectively). In our case, long-term exposed plants, grown in soil, presented the maximum TM at 1000 mg l^{-1} of Pb^{2+}, with tissues accumulating 65.9 and 2431.5 µg of Pb per g of dry weight. These data indicates that acute and chronic exposure to Pb can present the same pattern of damage.

Gichner *et al.,* (2008c) stated that the Comet assay probably did not represent a suitable method for monitoring genotoxicity of environmental pollutants *in situ*. Those authors justified their statement by claiming that performing the assay would be difficult due to the reduced availability of root's nuclei in plants growing for long periods in polluted soil and also to soil parts attaching to the roots. In our work, plants were grown in soil for nearly a month and we proved that the Comet

assay can be successfully applied to detect DNA damage in these conditions, even at low exposure doses, if the proper care is taken (*e.g.* rinsing the roots thoroughly, increasing the amount of tissue).

In order to evaluate Pb^{2+} effect in DNA content, cell cycle dynamics and DNA damage, FCM was used in leaves and roots exposed to this metal. Regarding the DNA content, we did not find significant variation in DNA content or ploidy level in any of the conditions tested ($P \leq 0.05$). Despite that in our case we did not find significant variation in DNA content, FCM has been successfully used to detect DNA content variation and ploidy level changes in plants exposed to toxicants; Monteiro *et al.,* (2010) reported a significant decrease in the DNA content of lettuce roots exposed to 100 µm of Cd while Citterio *et al.,* (2003) detected a ploidy level variation in hemp cultivated in soils contaminated with metals.

As for the cell cycle dynamics, the population of cells extracted from roots exposed to 2000 mg l^{-1} suffered a shift of around 20% from G_0/G_1 to G_2 (in respect to control) indicating a cell cycle arrest at the G_2 checkpoint. Cells are arrested at this checkpoint when DNA synthesis has been compromised, giving cells extra time to either repair the damage (O'Connell and Cimprich, 2005) or activate an apoptosis-like program. However, in some cases cells might continue to proliferate without completing the damage repair (Carballo *et al.,* 2006), giving rise to abnormal cells. We present proof of DNA damage due to Pb^{2+} exposure by Comet assay but also through the FPCV, thus reinforcing the hypothesis of cell cycle arrest due to DNA damage. The FPCV or CV of the G_0/G_1 peak has been used to detect clastogenic damage in maize (McMurphy and Rayburn, 1993) exposed to fly ash, in maize and wheat exposed to aluminium (Rayburn and Wetzel, 2002), in lettuce plants exposed to Cd (Monteiro *et al.,* 2010) and in pea plants exposed to Cr(VI) (Rodriguez *et al.,* 2011).

Comparing the FPCV and the TD/TM as DNA damage biomarkers, the latter proved to be more sensitive to Pb induced damage but the FPCV was the only marker to significantly correlate with the roots' Pb content ($P = 0.0073$ and $r = 0.966$). The TD and TM present a r below 0.800 and P values above 0.100. In leaves, the Pearson's correlation did not present significant relevance for any of the markers. These results together with previous reports of Wagner *et al.,* (1998) corroborate that the FPCV and the Comet assay markers measure different types of damage: while the FPCV translate chromosome brakes, the Comet assay detects DNA strand breaks, alkali-labile sites and incomplete excision repair sites of damaged DNA. With this, is understandable that the FPCV responds in a dose dependent manner (unlike the Comet assay) as the chromosome brakes seem to increase with the concentration of Pb. As mentioned before, the formation of DNA-DNA and DNA-protein links decreases the DNA migration in the Comet assay.

The data regarding Pb-induced MSI demonstrated that only for roots exposed to the maximum dose could MSI be observed, being indicative of defective functioning of the MMR system. Cenkci *et al.,* (2010) found that the genomic template stability of *Brassica rapa* was compromised when plants were exposed to Pb. Those authors also observed that Pb had negatively affected the template stability of leaves at all the dosages tested; on the other hand, we

only found evidence of Pb effect in roots exposed to the maximum dosage used. RAPDs are known for having low reproducibility, being prone to artifacts and producing spurious amplifications products (Atienzar and Jha, 2006) and these issues might justify why Cenkci and co-workers found Pb-induced genomic template instability even at low Pb dosages. Another possible explanation might be related with the inherent sensitivity of each species to Pb genotoxicity; it might be possible that *B. rapa* is more sensitive to or accumulates significantly higher amounts of Pb than *P.sativum*.

Interestingly, the results of the MSI evaluation are in agreement with those of the cell cycle dynamics and might explain, in part, the arrest at the G_2/M checkpoint observed in roots exposed to the maximum dosage, despite the fact that significant DNA damage could be detected at lower dosages. From all our results, it seems highly likely that for the cell cycle to be arrested a critical amount of DNA damage is required to activate the G_2/M checkpoint. This critical level might only be achieve after the DNA repair system is compromised and this condition was only verified in roots exposed to the highest dosage tested. With the data presented in this work, we propose a model for Pb-induced genotoxicity, based on our results (Figure 6)

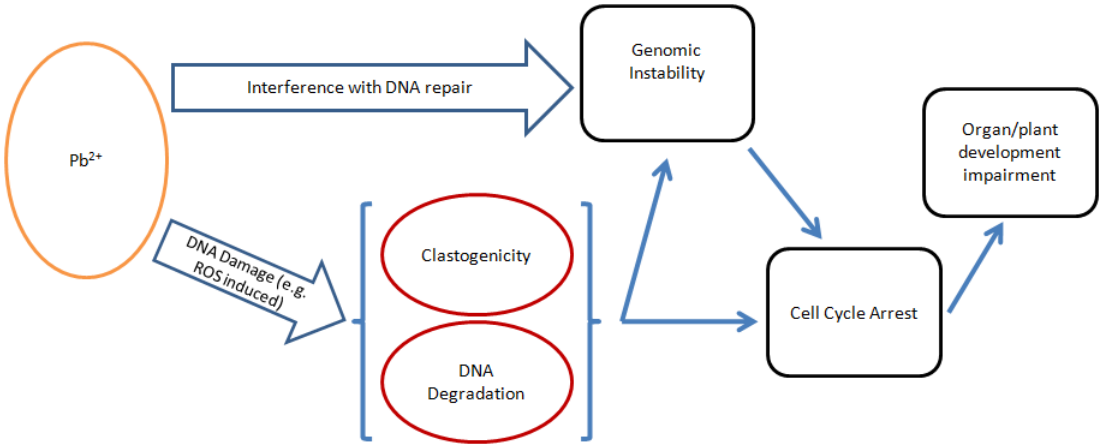

Figure 6 Functional model of Pb^{2+} genotoxicity

Taken all together, we report for the first time in plants that Pb can induce different types of DNA damage (DNA strand brakes, Chromosome brakes and MSI) which might be the cause for the arrest at the G_2/M checkpoint, observed in roots exposed to the highest dosage. Moreover, and also for the first time, it has been proved that exposure to Pb can lead to MSI, at least for roots exposed to the highest dosage tested, demonstrating that this metal interferes with the plant's MMR system. The suitability of the Comet assay to evaluate DNA damage in plants grown on soil was also clarified, contradicting previous suggestions that this technique might not be suitable to assess plants grown on soil (Gichner *et al.,*2008c). Finally, as we found evidence of Pb-induced damage in doses as low as 20 mg l^{-1} (the highest admitted concentration by the European Union for this metal in agricultural purpose water) the data presented provides critical information of ecological and regulatory importance, suggesting that it might be advisable to recommend more restrictive values for this metal

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CHAPTER II-2

CR(VI) INDUCES DNA DAMAGE, CELL CYCLE ARREST AND POLYPLOIDIZATION: A FLOW CYTOMETRIC AND COMET ASSAY STUDY IN *PISUM SATIVUM*

Chapter published as an original article to an SCI journal

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Abstract

Chromium(VI) is recognized as the most toxic valency of Cr, but its genotoxicity and cytostaticity in plants is still poorly studied. In order to analyze Cr(VI) cyto- and gentotoxicity, *Pisum sativum* L. plants were grown in soil and watered with solutions with different concentrations of Cr up to 2000 mg l^{-1}. After 28 days of exposure, leaves showed no significant variations in either cell cycle dynamics or ploidy level. As for DNA damage, flow cytometric (FCM) histograms showed significant differences in the full peak coefficient of variation (FPCV) values, suggesting clastogenicity. This is paralleled by the Comet assay results, showing an increase in DNA damage for 1000 and 2000 mg l^{-1}. In roots, exposure to 2000 mg l^{-1} resulted in a cell cycle arrest at G_2/M checkpoint. It was also verified, that under the same conditions, 40% of the individuals analyzed suffered a polyploidization having both 2C and 4C levels. DNA damage analysis by Comet assay and FCM revealed dose-dependent increases in DNA damage and FPCV. Through this, we have unequivocally demonstrated for the first time in plants that Cr exposure can result in DNA damage, cell cycle arrest and polyploidization. Moreover, we critically compare the validity of Comet assay and FCM in evaluating cytogenetic toxicity tests in plants and demonstrate that the data provided by both techniques complement each other and present high correlation levels. In conclusion, the data presented provides a new insight on Cr effects in plants in general and supports the use of the parameters tested in this study as reliable endpoints for this metal toxicity in plants.

Keywords: cell cycle; chromium; comet assay; flow cytometry; genotoxicity; *Pisum sativum.*

Introduction

Heavy metal pollution is a relatively recent concern but an ancient problem as technological advances like the discovery of fire or ore-mining and more importantly the industrial revolution, have contributed to continuously and exponentially increase metal pollution in the environment. Even worse, as heavy metals are not degraded by living organisms, they can easily accumulate to harmful levels. From the many forms of heavy metal toxicity, genotoxicity poses one of the major threats to organisms. Damage to the DNA can have severe repercussion to cells like mutagenesis or de-regulation of the cell replication machinery, among others, which could ultimately result in tumorigenesis and death. While most genotoxic heavy metals do not cause DNA damage directly, rather acting through ROS (Reactive Oxygen Species) formation and/or interference with DNA repair, Chromium (Cr) is a special case. Once inside the cell, Cr (VI) is sequentially reduced (through Cr V and IV) to Cr (III) while producing ROS in the process. It is the latter Cr valence which contrarily to other genotoxic heavy metals interacts primarily and directly with DNA, forming DNA adducts (Beyersmann and Hartwig, 2008).

The role of Cr as an environmental pollutant is critical; Cr occurs naturally in the environment being one of the most abundant elements on the Earth's crust (Kotas and Stasicka, 2000). Another important source of Cr presence in the environment is due to anthropogenic activities, like tanning and chrome-plating, making this heavy metal a common contaminant of water and soils (Babula *et al*., 2008). Among the different oxidations states in which Cr can be found, Cr(III) and Cr(VI) are the most commonly observed and stable (Avudainayagam *et al*., 2003) and it has been demonstrated that Cr(VI) is more mobile than Cr(III) and Cr from tannery sludge, causing greater toxic effects at lower concentrations (Lopez-Luna *et al*., 2009).

In animals and yeast, Cr(VI) has been extensively studied, and it has been demonstrated to induce cell cycle arrest and as having carcinogenic effects (e.g. Zhang *et al*., 2001, O'Brien *et al*., 2002, Salnikow and Zhitkovitch, 2008). Despite the critical importance of Cr toxicity, we are still far away from having from plants, the same level of understanding that exists in other eukaryotes about the mechanism and effects of Cr genotoxicity. From the little that is known (see table 1 for a literature survey of Cr genotoxicity in plants) most of the research presented demonstrates that Cr generates chromosomal aberration and micronuclei formation. There are also 2 articles showing that Cr can cause point mutation and DNA degradation, although the purpose of the latter was to establish a technique (Comet Assay) to study plant genotoxicity rather than to address Cr genotoxicity. It is therefore urgent to study and produce more data on Cr genotoxicity in plants.

To perform a comprehensive and thorough evaluation of Cr genotoxicity it is imperative to use sensitive and robust techniques which provide clear and unbiased data. Flow cytometry (FCM) and single cell gel electrophoresis (or Comet assay) are two outstanding techniques to study genotoxicity, which have been increasingly used on Cr-induced toxicity in animal research (*e.g*. Cavallo *et al*., 2010, Oliveira *et al*., 2010, Zhang *et al*., 2008)

Table 1. Literature survey of genotoxicity studies in plants.

Species	Reference	Dose	Technique	Effects
V. faba	Chandra *et al*., 2004	Tannery solid waste	Cytogenetic	Chromosomal and mitotic aberration
B. napus	Labra *et al*., 2004	$K_2Cr_2O_7$ (10 to 200 mg l^{-1})	AFLP, SAMPL, DNA methylation analysis	Methylation changes, Mutation
A. thaliana	Labra *et al*., 2003	$K_2Cr_2O_7$ (2, 4 and 6 mg l^{-1})	AFLP	DNA mutation
C. sativa	Citterio *et al*., 2003	$K_2Cr_2O_7$ (25µg g^{-1} and 50 µg g^{-1} soil)	FCM	ND
T. repens	Citterio *et al*., 2002	Contaminated soils from a steelworks- Up to 4810 mg kg^{-1} soil	AFLP, FCM	Mutation, DNA decrease
V. faba	Wang, 1999	Cr (contaminated soils)	MN	Dose-related increase of MN
A. cepa	Matsumoto *et al*., 2006	Tannery effluent	Cytogenetic	Chromossomal aberration
V. faba	Koppen and Verschaeve, 1996	$K_2Cr_2O_7$ (Up to 10^{-3}M)	Comet assay	Increase in %Tail DNA, Tail moment and Tail length
Tradescancia sp. ***V. Faba***	Knasmuller *et al*., 1998	$CrCl_3$, CrO_3 (from 0.75 to 10 mM)	MN	Dose-related increase of MN for *Tradescancia*, ND in *V. faba*

FCM is a rapid and multiparametric technique, *i.e*., with a single analysis it can provide information on variations in DNA content; polyploidization; evaluate cell cycle dynamics and also DNA damage. In plants, Pfosser *et al*., (1995) detected differences in DNA content as small as 1% in aneuploid wheat-rye lines, on a study developed to evaluate the sensitivity of FCM. Relatively to DNA damage, Rayburn and Wetzel (2002) correlated the coefficient of variation of the G_0/ G_1 peak with chromosomal aberration in aluminum exposed plants, as this parameter is able to detect broken and rearranged chromosomes in daughter cells. Monteiro *et al.,* (2010) also detected an increase in the full peak coefficient of variation (FPCV) of the G_0/ G_1 peak of lettuce plants exposed to Cd.

The Comet assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins *et al*., 2008). Due to its simplicity, sensitivity and the small number of cells/nuclei required to obtain robust results (Hattab *et al*., 2009), it has been extensively applied to evaluate genotoxicity. In plants, Comet assay has been proven to be very useful to study genotoxicity of heavy metals (*e.g.* Hattab *et al*., 2009, Gichner *et al*., 2006, Gichner *et al*., 2008). It has even been used to evaluate the effect of Cr in root cells of *V. faba* (Koppen *et al*., 1999) although the objective of the work was to test the applicability of Comet assay in plants and only a short term exposure (2 h) to Cr was used.

The objective of the investigation presented here was to characterize the genotoxicity of Cr(VI) in the model crop species *P. sativum*. In order to fulfill this aim, pea plants were exposed to different concentrations of Cr (VI) solutions. Cr(VI) concentrations were based on the maximum admitted level in some European Union countries for agricultural purpose waters (20 mg l^{-1} for total chromium) and increased up to 2000 mg l^{-1} (value detected in waste waters coming from chrome tanning process- Iyer and Mastorakis, 2006). Roots and leaves were collected and the level of DNA damage, cell cycle dynamics and ploidy level were determined. This information will provide a new insight on Cr(VI)'s genotoxicity in plant organs. The potential use of the techniques and the endpoints measured as genotoxicity biomarkers for heavy metal induced stress is also discussed.

Material and Methods

Plant material

Pea seeds (*Pisum sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sawed in pots containing a peat:perlite mixture (4:1). Plants were grown during 28 days at 24ºC ± 1ºC, under light intensity of 200 μmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark).

Cr treatment consisted in watering plants (at least 25 per condition) during the aforementioned period of 28 days, twice per week, with 100 ml of a 1:10 Hoagland's solution containing the following concentrations of $K_2Cr_2O_7$: 0; 20; 200; 1000 and 2000 mg l^{-1}. Afterwards plants were collected, rinsed thoroughly to remove substrate adhered to roots and tissues were sampled for analysis. At least two independent experiments were performed for each parameter.

Chromium analysis

The total Cr concentration in control and Cr-treated plants (at least 3 plants per condition, with 3 replicates per sample) was verified by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France). Accumulation of Total Cr was determined in shoots and roots dried to constant weight at 60ºC. Prior to drying roots were washed thoroughly with distilled water to remove Cr adsorbed to the root surface. Dried tissues were treated according to Evans and Bucking (1976); in brief, dried tissues were placed in a crucible at 530 ºC during 14h in order to reduce samples to ashes. Then, ashes were digested in three steps by successively adding HCl dilutions (decreasing concentration in each step) and heating. After this, samples were filtered by vacuum suction using Whatman paper filter nº1 and a Buchner funnel. The crucible and then the funnel were washed with 10% HCl (v/v) to minimize loss of sample. Finally, ultra pure water was added until final volume of 14 ml was obtained and samples were analyzed by ICP-AES.

Flow cytometry

For FCM analyses, root tips (2 mm from the root apex) and leaves were treated as follows: Tissue was placed on a Petri dish and chopped in 1 ml of the Woody Plant nuclei extraction Buffer (WPB- Loureiro *et al*., 2007). Nuclei suspension was collected and filtered through a 55 µm nylon mesh to remove large debris. Afterwards, 50 µg ml^{-1} of propidium iodide (PI) and 50 µg ml^{-1} of RNase were added to label DNA and degrade RNA respectively. After 5 min incubation on ice, samples were analyzed in a Coulter Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. The results were acquired using the SYSTEM II software version 3_0 (Coulter Electronics). Prior to analysis, the instrument was checked for linearity with fluorescent beads (Coulter Electronics), and the amplification settings were kept constant throughout the experiment.

The proportion of cells in each of the cell cycle phases, the full peak coefficient of variation (FCPV) of the G_0/G_1 peak and the coefficient of variation (CV) of the G_0/G_1 were collected from the fluorescence intensity (FL) histograms (at least 5 plants per condition). For cell cycle analysis, flow cytometric files were transformed to single histograms files with WinMDI ver 2.8 software (Freeware by Joe Trotter of the Scripps Institute, La Jolla, CA.) and then analyzed using Cylchred software (Freeware developed by Terry Hoy of the University of Cardiff). Resultant data was treated using Microsoft Office Excel 2007.

For nuclear DNA content determination (10 plants per condition), *Vicia faba* cv Inovec, containing a DNA content of 26.90 pg per 2C, was used as an internal standard in order to determine the DNA content of *P. sativum* plants, as described by Loureiro *et al*., (2007). At least 5000 nuclei were analyzed in each sample.

Comet assay

Roots and leaves were processed as described by Gichner *et al*., (2008) for the alkaline Comet assay procedure, with some modifications. At all moments, the procedures were conducted under dim light to avoid light-induced DNA strand brakes. Leaves or roots were placed in a Petri dish kept on ice altogether with 20 ml ice-cold Tris buffer (400mM, pH 7.5) and gently sliced with a razor blade. Frosted edge microscope slides were covered with 1 % normal melting point agarose (NMP) at 50ºC, dried over night and kept covered in the dark, until usage. The nuclei suspension was placed in the slide together with 1% low melting point agarose (LMP) prepared in PBS, mixed by gently pippeting, followed by covering the mixture with a cover slip and incubation on ice for at least 5 min. After this, the cover slip was removed, a layer of 0.5% LMP was added and a new cover slip was placed on the slide, which was incubated for 5 min on ice.

A parallel test of the unwinding and electrophoresis time was assessed and the optimal times for *P. sativum* were chosen (data not shown). Nuclei were unwinded by placing the slides in

alkaline electrophoresis buffer for 15 min prior to electrophoresis at 0.74 V cm^{-1} for 15min. Afterwards, the slides were rinsed three times with 400 mM Tris buffer (pH 7.5), air-dried and stored until further use.

For analysis, the slides were hydrated in distilled water for 10min, stained with 80 µl ethidium bromide (20 µg ml^{-1}) for 5min and excess stain was removed by rising in distilled water three times. The slides were then covered with a cover slip and analyzed with a Nikon Eclipse 80i with an excitation filter of 510-560 nm and a barrier filter of 590 nm.

Comet images were analyzed using CASP v1.2.2 software. At least three slides per condition (each slide prepared from a different individual) were evaluated per treatment and at least 25 nuclei per slide were exanimate. The % Tail DNA (TD) and the Tail moment (TM) values were recorded and compared. Values presented are given as the average of medians ± SE of at least 75 nuclei per condition.

Statistical analysis

Statistical significance of treatments was assessed by One-Way ANOVA with a post-hoc Holm-Sidak multiple comparison test, using SigmaStat 3.5 for WINDOWS (SPSS Inc., Chicago, IL, USA). Pearson's correlation among the endpoints tested was performed using SigmaPlot for Windows ver. 11.0 (Systat Software inc).

Results

Overall characterization of plant growth and morphology

Chromium exposure affected plant morphology mostly starting from 1000 mg l^{-1}. Roots became progressively thin, brittle and brownish. Leaves, though in a lesser extent than roots, presented signs of deterioration mostly at the two highest concentrations. The leaves exposed to those concentrations acquired a slightly paler coloration and became frailer in comparison to control leaves. Relatively to plant growth, Figure 1 illustrates the values obtained for roots and shoots of control and exposed plants. In roots significant growth inhibition ($P \leq 0.05$) was observed at 2000 mg l^{-1} (11.86 ± 4.514 cm) corresponding to a decrease of 42% in respect to control (19.13 ± 5.833 cm). For shoots, on the other hand, little differences ($P > 0.05$) were observed among concentrations, being the maximum value obtained at 20 mg l^{-1} (42.56 ± 5.109 cm) and the lowest at 2000 mg l^{-1} (33.14 ± 9.032 cm).

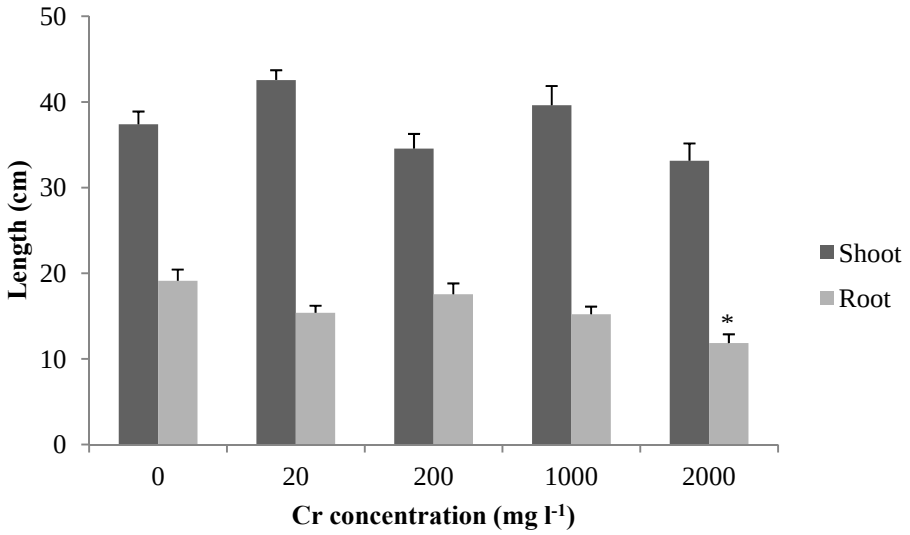

Figure 1 Shoots and roots growth (in cm) after 28 days of exposure to Cr. Values are given as mean ± SE of 20 individuals. (*) significantly different from control ($P \leq 0.05$)

Chromium content in organs

Total chromium content in roots and leaves of plants exposed for 28 days to Cr is presented in Figure 2. In exposed plants, the amount of Cr in both tissues was significantly higher than for control ($P \leq 0.05$) and increased with increasing external Cr concentrations. Roots accumulated in general 2 to 4 times more Cr than leaves; the highest difference being found for 200 mg l^{-1}. The maximum values of Cr contents were observed at 2000 mg l^{-1} with 1236.8 ± 125.34 and 529.5 ± 68.11 µg Cr g^{-1} DW (mean ± SE) for roots and leaves, respectively.

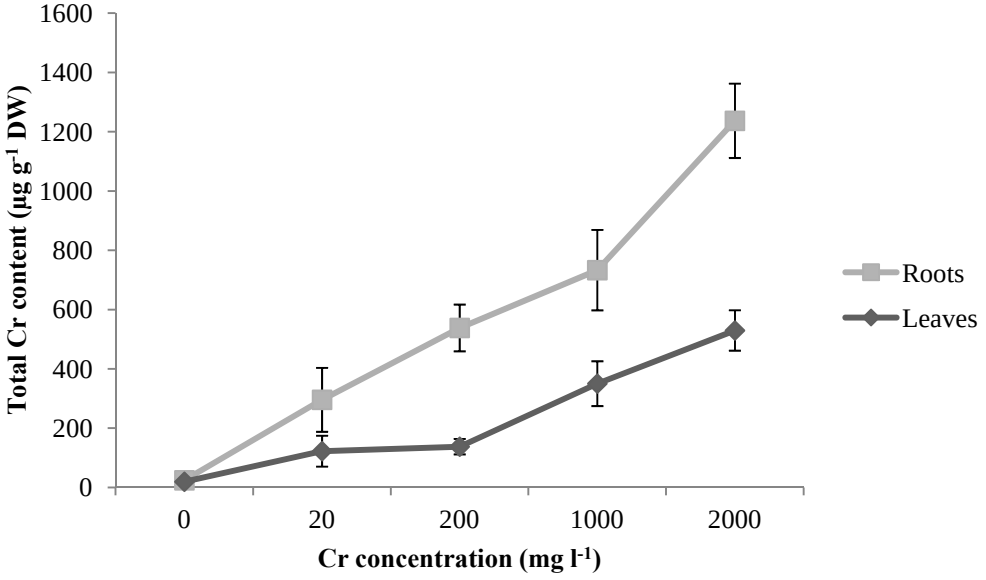

Figure 2 Total chromium content in roots and leaves exposed for 28 days to given concentration (mg l^{-1}). Results are given as mean ± SE of 3 replicates per condition. All values are significantly different from control ($P \leq 0.05$).

Flow cytometry

The flow cytometric analysis of nuclei extracted from both tissues showed no statistical variation in either volume or granularity ($P > 0.05$), independently of the concentration tested (data not shown).

The FL histogram for control plants displayed a main peak, corresponding to nuclei at G_0/G_1 (84 % and 45 % of the events, for leaves and roots respectively) and a secondary peak corresponding to G_2 (around 8% and 40 %, for leaves and roots respectively). Control plants presented CV (FCM quality measurement) of 2.30 ± 0.138 and 3.40 ± 0.249, leaves and roots respectively, while the highest values were observed at 2000 mg l^{-1}, with 3.04 ± 0.230 and 3.52 ± 0.470, leaves and roots respectively (table 2).

In roots, significant increases in G_0/G_1 peak FPCV values were observed for 1000 mg l^{-1} (1.27 fold) and 2000 mg l^{-1} (1.21 fold) in respect to the remaining concentrations ($P \leq 0.05$). Leaves exposed to these concentrations also showed a significant increase in FPCV values (1.16 and 1.20 fold for 1000 and 2000 mg l^{-1}, respectively), in respect to control ($P \leq 0.05$).

Table 2. Coefficient of variation (CV) and full peak coefficient of variation (FPCV) of roots and leaves of plants exposed to Cr. Values are given as mean ± SD of 5 individuals. (*) significantly different from control ($P \leq 0.05$).

	Cr [mg l^{-1}]	CV	SD	FPCV	SD
Leaves	0	2.30	0.138	3.24	0.196
	20	2.52	0.428	3.62	0.430
	200	2.41	0.298	3.62	0.063
	1000	2.68	0.071	3.77*	0.098
	2000	3.04	0.230	3.90*	0.123

	Cr [mg l^{-1}]	CV	SD	FPCV	SD
Roots	0	3.44	0.249	4.39	0.253
	20	3.34	0.451	3.94	0.181
	200	2.29	0.243	4.88	0.599
	1000	3.09	0.254	5.58*	0.341
	2000	3.52	0.474	5.29*	0.280

Roots exposed to 2000 mg l^{-1} of Cr presented an extra ploidy level (4C), along with the normal 2C verified for all other conditions (Figure 3a). Of the individuals analyzed (10 per condition), 40% presented both 2C and 4C ploidy level (Figure 3b). However, the leaves of these individuals only had 2C ploidy level.

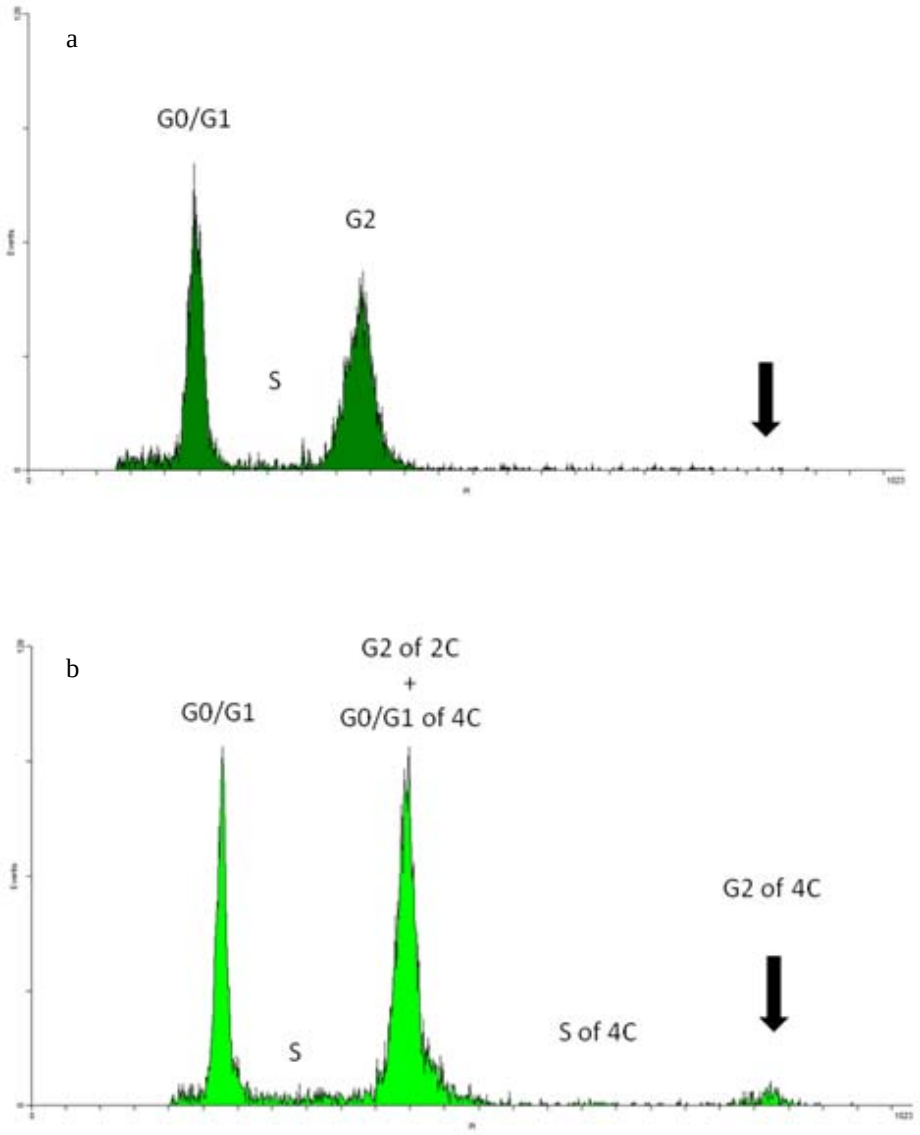

Figure 3 Fluorescence histograms of control (up) and 2000mg l^{-1} (bottom) Cr (VI) exposed roots. Values are given in channels (X axis) and nº of events (Y axis). The arrow indicates the position of extra peak in the bottom histogram (not present in control).

As for variation of DNA content (besides the aforementioned extra ploidy level) no significant differences in DNA content were found between control and any of Cr treated tissues (data not shown), with variations below 3% of the 9.02 pg of DNA per 2C presented by control plants.

Putative cytostatic/mitogenic effects were also evaluated. In leaves, the profile of cell cycle progression showed little variation among any of the conditions assayed ($P > 0.05$) (data not shown). Contrarily, roots exposed to 2000 mg l^{-1} Cr presented a significant decrease ($P \leq 0.05$) of the proportion of cells at G_0/G_1 (a decrease of around 20%) when compared to all other

concentrations (figure 4). A significant increase in the % of cells at G_2 of roots of the same condition (22% increase in respect to control) was also verified ($P \leq 0.05$).

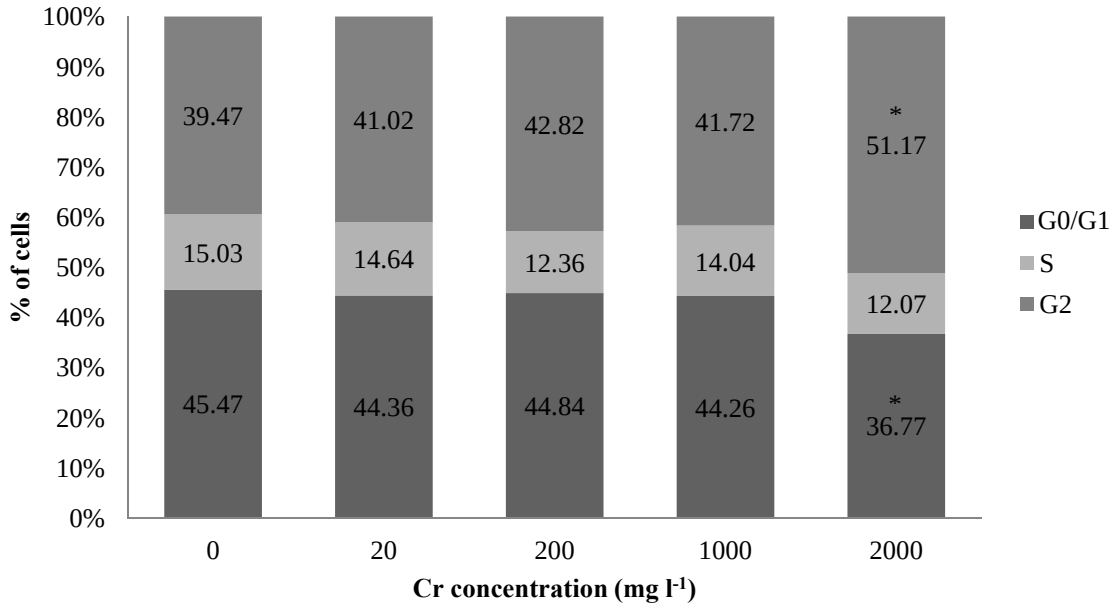

Figure 4 Cell cycle dynamics of roots exposed to Cr. Values given are the mean % of cells in each of the cell cycle phases of 5 individuals per condition. (*) significantly different from control ($P \leq 0.05$)

Comet assay

Exposure to Cr(VI) caused significant DNA damage as determined by Comet assay, in both tissues (Figure 5 and 6) using the Tail Moment (TM) and % of Tail DNA (TD). The TD (average median ± SE) of leaves exposed to 1000 mg l^{-1} (13.68 ± 2.341) and 2000 mg l^{-1} (18.07 ± 3.543) was significantly higher ($P \leq 0.05$) than that of control 6.99 ± 0.842 (Figure 5). This corresponded to 2 (1000 mg l^{-1}) and 2.5 (2000 mg l^{-1}) fold increase in respect to control. For 20 mg l^{-1} and 200 mg l^{-1} significant variations in TD were not observed ($P > 0.05$). The TM presented a similar profile to TD (Figure 5) varying from 4.08 ± 0.83 control leaves to 27.60 ± 7.963 for 2000 mg l^{-1} exposed leaves.

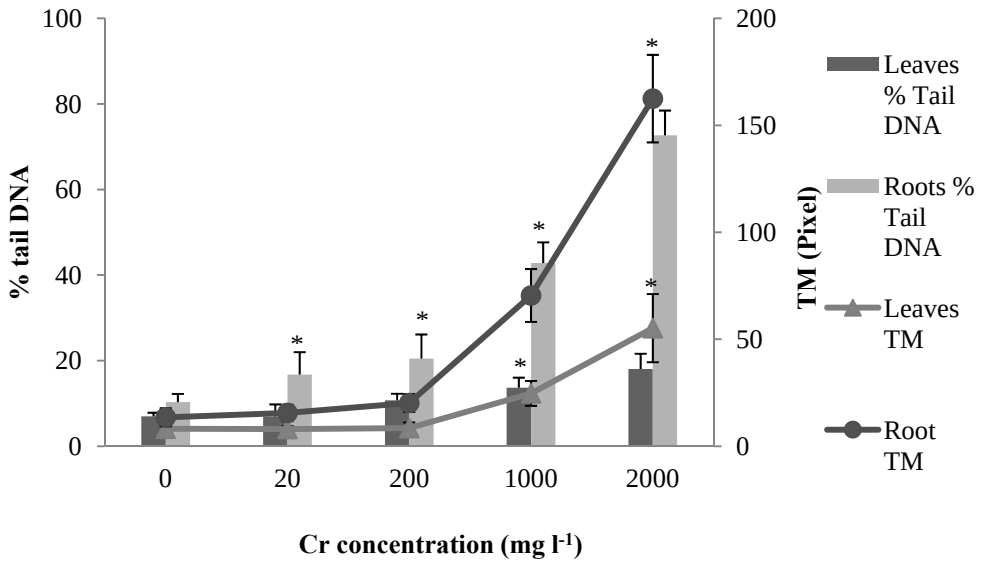

Figure 5 % of Tail DNA and TM of roots and leaves exposed to given Cr concentrations. Values are given as mean ± SE of at least 3 replicates with at least 25 nuclei per replicate. TM values are given in arbitrary units (right axis). (*) significantly different from control ($P \leq 0.05$)

The damage observed in roots DNA was higher than that observed in leaves. Exposure to Cr (any concentration) resulted in a significant increase in TD (Figure 5, 6a). The variation of TD ranged between 10.33 ± 1.890 for control (average median ± SE) to 64.96 ± 4.869 for 2000 mg l^{-1} (Figure 5, 6e), an increase of around 6 fold. The TM presented similar variation to TD, ranging between 13.50 ± 4.221 (control) and 149.917± 20.750 for roots exposed to 2000 mg l^{-1} (Figure 5).

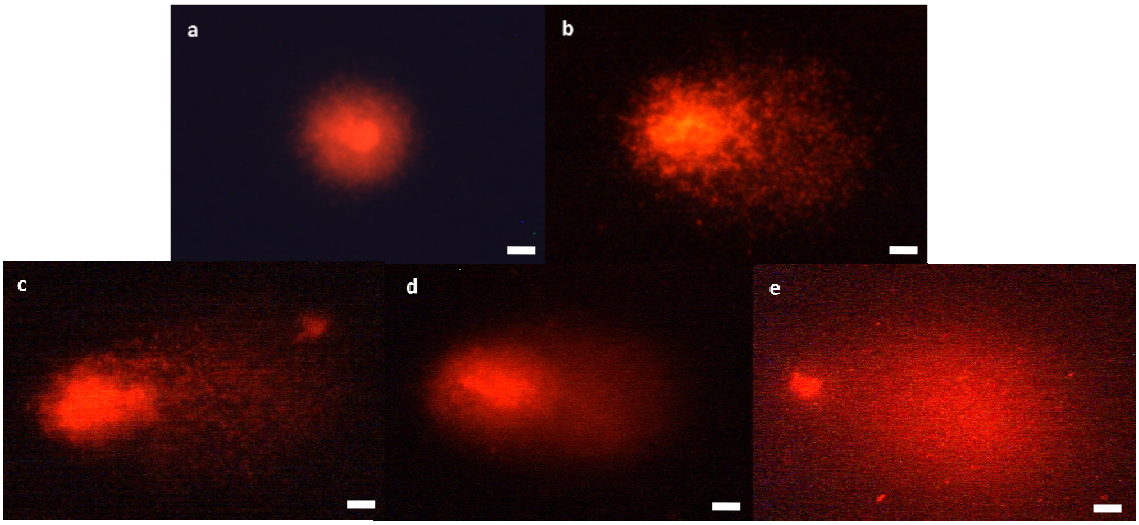

Figure 6 Comet assay representative images of nuclei extracted from roots: a) control, b) 20 mg l^{-1}, c) 200 mg l^{-1}, d) 1000 mg l^{-1} and e) 2000 mg l^{-1}. Magnification scale bar (-) = 40×

Discussion

Chromium toxicity in plants has not received the same attention as other heavy metals, despite its known genotoxicity and carcinogenicity. Although there is a good level of understanding about the toxicity mechanism and effects of this heavy metal in animals, in plants we are still far behind. Within the little available information on Cr(VI) induced genotoxicity in plants (see table 1 for review) it was demonstrated that Cr may cause cytogenetic abnormalities such as micronuclei formation, chromosomal aberration and point mutations (AFLP instability). The Comet assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins *et al*., 2008) and has been extensively applied to monitor genotoxicity in animals. In plants, Comets have successfully diagnosed DNA damage induced by different types of contaminants such as Pb, Cu or organic compounds (*e.g.* Hattab *et al*., 2009, Gichner *et al*., 2006, Gichner *et al*., 2008) being that most of these applications use the TM as a measurement of DNA damage. However Collins *et al*., (2008) suggested that although this parameter may be more informative with small levels of damage; TD covered the widest range of damage. Moreover, TD is linearly related to break frequency allowing better inter-laboratory comparison (Collins *et al*., 2008). To clarify the contribution/sensitivity of each of these two endpoints in Cr-induced genotoxicity, changes in both TD and TM were statistically compared. Results indicate that they conveyed a similar type of information; moreover, these parameters showed a high Pearson correlation coefficient $r = 0.929$ ($P \leq 0.001$) for leaves and $r = 0.983$ ($P \leq 0.001$) for roots, supporting that either can be used with confidence. Notwithstanding, TD presented better PC with both FPCV and tissue Cr accumulation than the TM and so, at least in this case, it might be advisable to use TD over TM.

Our data has demonstrated that Cr exposure causes significant DNA damage in roots (at all concentrations) and leaves (only for concentrations ≥ 1000 mg l^{-1}). When regarding these results together with the data of Cr accumulation, it is understandable that roots presented higher DNA damage than leaves. Another conclusion that might be taken from this data is that Cr transport/accumulation in leaves is minimal up to 200 mg l^{-1}. The Cr content values in pea roots presented here are within the range reported by Koppen and Verschaeve (1996) in *V. faba* plants exposed for 2h to 10^{-3}M (around 300 mg l^{-1}) of Cr(VI), which showed an increase of 1.4 fold with respect to control roots (the amount of Cr accumulated by leaves was not determined).

In Cd-toxicity studies (Gichner *et al*., 2004, Gichner *et al*., 2008) roots presented higher toxicity levels than leaves and the authors justified these differences with a better anti-oxidant defense system in leaves, which might protect the nuclear DNA. They also argued that the lower levels of Cd accumulated by leaves might be another explanation for these differences. In our Cr-exposed plants, we demonstrate that independently of the anti-oxidative stress status, a positive Pearson correlation ($r = 0.928$, $P \leq 0.05$ for both tissues) is found between Cr accumulated and DNA damage for both tissues. In fact, when both organs accumulate similar levels of Cr, the range of variation in TD is not significantly different ($P > 0.05$). For instance, Cr accumulated by roots exposed to 200 mg l^{-1} is similar to that accumulated in leaves of plants exposed to 2000 mg l^{-1} and the values of TD in these organs are also similar (20.5 and 18.7%, respectively).

Another interesting point that can be highlighted when comparing our results with those of other heavy metals is that in our case DNA damage increased linearly with Cr accumulation while for other heavy metals (Gichner *et al*., 2008, Rucinska *et al*., 2004) beyond a certain dose the level of DNA damage decreased even as the heavy metal dose increased. This was justified by low DNA migration due to DNA-DNA and DNA-Protein complex formation promoted by high doses of heavy metals.

All of the above stated, fits within the proposed mechanism of Cr direct interaction with DNA (Beyersmann and Hartwig 2008), in which Cr(VI) is reduced inside the cell to Cr (III), which then forms Cr-DNA adducts. It is therefore likely that, as was demonstrated for other organisms, in plants, this heavy metal has the ability to directly interact with DNA.

Flow cytometry was used to assess cyto- and genotoxicity caused by Cr exposure. Our results demonstrate a change in cell cycle dynamics of roots exposed to the highest concentration tested. The decrease observed in the % of G_0/G_1 cells of roots is accompanied by an increase of the % of cells in G_2 (20% variation in respect to control), indicating cell cycle arrest at the G_2/M checkpoint. Carballo *et al*., (2006) stated that cell cycle arrest results in a slowdown of cell division, which in turn, would turn into growth impairment. Our findings are in agreement with this; roots exposed to 2000 mg l^{-1} besides showing cell cycle arrest were significantly shorter (42%) than control roots. Hattab *et al*., (2009) also found growth impairment (of around 50%) in roots of pea exposed to high doses of Cr, which further adds to the thesis of Cr induced growth slowdown. This is highly likely to be due to the fact that only at this dosage, enough Cr was accumulated inside the roots to cause the effect observed. This cell cycle arrest at G_2, not only demonstrates that Cr directly or indirectly affects the G_2/M checkpoint but also, together with the observed root growth reduction, supports a functional model (Figure 7) which is consistent with the current theories of root elongation that involve both cell division and expansion models (West *et al*., 2004).

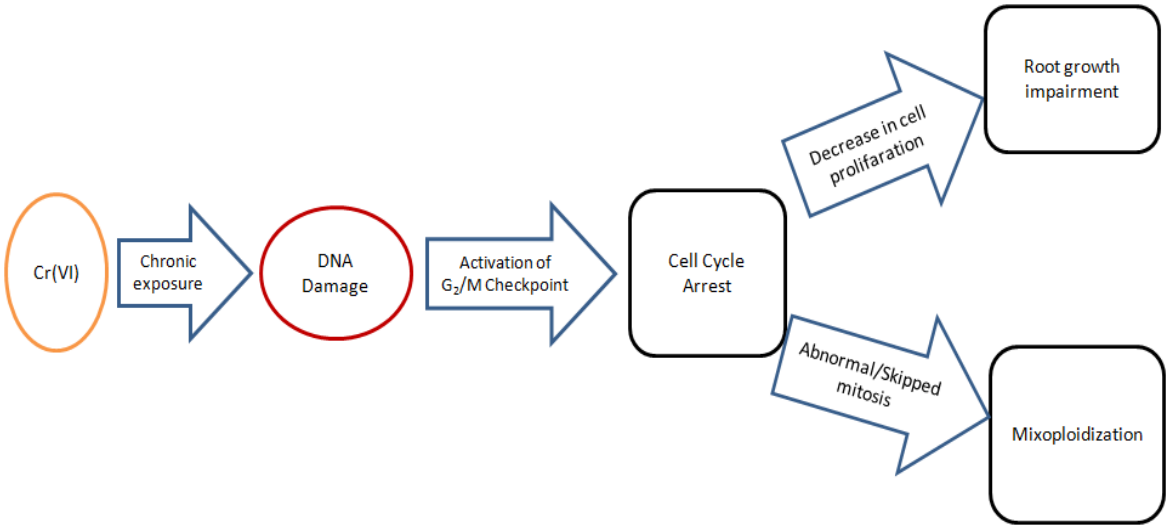

Figure 7 Functional model of Cr(VI) genotoxicity in plants.

Unlike root tips (which have high levels of actively dividing cells), leaves have only residual cells with meristematic activity. Therefore, in most cases, cell cycle distribution of leaves differs from that of root tips, as usually the ratio G_0/G_1:G_2 is much higher when compared to the G_0/G_1:G2 ratio observed in root tips. Secondly, what was said for roots exposed to lower Cr concentrations is also valid for leaves, i.e. the amount of Cr accumulated by leaves is, at all times (except for control), at least 2 fold lower than what is observed in roots of the same condition. This in turn might justify the absence of cell cycle arrest and/or polyploidization in the leaves of plants exposed to 2000 mg l^{-1}, where the level of Cr accumulated is significantly lower than that accumulated by roots ($P \leq 0.001$).

An arrest of cell cycle at the G_2/M checkpoint is part of the cell strategy to cope with DNA damage, giving the cell extra time to either repair the damage (O'Connell and Cimprich, 2005) or activate an apoptosis-like program. In some cases, cells might continue with proliferation without completing the damage repair (Carballo *et al*., 2006) giving rise to cells with genomic abnormalities. Our data provides evidence of DNA damage as both Comet assay and the FPCV values, which have been used to detect clastogenic and genotoxic effect in plants (Rayburn and Wetzel, 2002, Monteiro *et al*., 2010), were significantly higher in plants exposed to Cr.

Further prove of the genotoxic effect of Cr was found in roots exposed to 2000 mg l^{-1}, in which 40% of the individuals analyzed were mixoploid presenting diploid and tetraploid cells. Similar results were obtained by Citterio *et al*., (2002) in hemp plants exposed to heavy metals. These authors detected mixoploidy in roots and leaves, up to 16C. They hypothesized that the increase in DNA content observed could be a part of the plant strategy to overcome heavy metal toxicity. We find this to be unlikely; there is plenty of proof that heavy metals are genotoxic and cytotoxic to plants (*e.g.* Gichner *et al*., 2008, Citterio *et al*., 2003). In addition, it is not foreseeable that increasing the quantity of DNA (that is most certainly damaged) could be a strategy to cope with this type of toxicity. In the light of the results that we have presented here, it is more plausible that DNA/ploidy level increase is caused by a defective function of mitosis apparatus caused (directly or indirectly) by heavy metals. Supporting this is the fact that the higher levels of DNA damage assessed by Comet assay were observed in the same concentration in which the cell cycle arrest and the ploidy level change was also verified.

It is an important fact that the highest dose that was tested in our assay presented such deleterious effects to leaves and to a higher extent, in roots. Despite the fact that Cr(VI) levels in water for human consumption are several orders of magnitude lower, the values used in our assay can be found in water bodies contaminated by leachates and in wastewaters from tannery industries. In fact, Iyer and Mastorakis (2006), reported levels of Cr(VI) of 2,000 mg l^{-1} and in extreme cases up to 5,000 mg l^{-1} in wastewaters of leather tannery industries. Even though we did not test such an extreme concentration as 5,000 mg l^{-1}, the results observed in plants exposed to 2,000 mg l^{-1} are a huge indicator of the genotoxic proprierties of this heavy metal.

Relatively to variations in DNA content (besides the aforementioned mixoploidy), we did not find significant differences among any of the conditions tested, in either leaves or roots. Citterio

et al., (2002) reported a significant decrease of DNA content in *T. repens* shoots exposed to 25 and 50 ppm of Cr(VI). Unfortunately, the data presented by those authors presents several drawbacks which make their results questionable. Firstly, the authors used DAPI as the DNA fluorochrome which is not the most suitable for DNA content determination due to the fact that it preferably binds to AT rich regions. DNA intercalating dyes as PI, should be preferred for DNA content determination as these dyes can bind stoichiometrically to DNA, giving a better estimation of DNA content. Furthermore, when presenting FCM data, it is important to provide de CV values of the G_0/G_1 FL peaks, the standard of quality which guarantees unbiased data (Loureiro *et al*., 2006a). Citterio *et al.,* (2002) reported high levels of debris in plants exposed to Cr, but failed to present the CV of those samples. CV values for plant analysis have been deemed as acceptable when below 5% (Galbraith *et al*., 2002); when this is not verified, results should be regarded carefully. High CV, when accompanied by high levels of debris, usually point to factors (like cytosolic compounds) that might interfere with fluorescence emission and consequently with DNA content quantification (Loureiro *et al*., 2006b). In our case, the CV values of all our samples were well below the 5% acceptance criterion and the level of debris were very low, as it can be seen in (Figure 3). Caution must be exerted when reporting variations of DNA content in plants. Price and Johnston (1996) reported the influence of light on DNA content of *H. annuus* only to be proved later on by Price *et al*., (2000) to be an artifact caused by cytosolic compounds rather than real variations of DNA content.

In conclusion, we have demonstrated here for the first time in plants, the cytostatic effect of Cr(VI) as cell cycle arrest at the G_2/M checkpoint. Moreover, we correlate this arrest with DNA damage detected by Comet assay and FCM, which also detected polyploidization in roots exposed to 2000 mg l^{-1}. We have also shown that Comet assay is more sensitive in DNA damage detection than FCM. Notwithstanding, the data provided by both techniques complement each other and allowed us to provide the first comprehensive study of Cr(VI) genotoxicity in plants, proving that these techniques can be used to measure endpoints for genotoxic evaluation.

Finally, by detecting significant DNA damage in roots exposed to 20 mg l^{-1} of Cr, which is the highest admitted concentration by the European Union in agricultural purpose water, we have provided critical information suggesting that it might be advisable to recommend more restrictive values for this metal. Also, the results obtained in plants exposed to 2000 mg l^{-1} of Cr are a huge warning to waste water management, as these values can be found in waste waters that can have highly negative repercussion to the ecosystem.

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CHAPTER II-3

CR(VI) INDUCED MICROSATELLITE INSTABILITY IN PLANTS USING *PISUM SATIVUM* AS A MODEL

Chapter submitted as an original article to an SCI journal

Rodriguez E, Azevedo R, Moreira H, Fernandes P, Souto L and Santos C (2011). Cr(VI) induced microsatellite instability in plants using *Pisum sativum* as a model. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. (submitted).

Abstract

Chromium is an environmental pollutant known to be carcinogenic and genotoxic in animals. In plants, on the other hand and despite the recurrence Cr contamination of ecosystems, the genotoxicity of this metal has not received the same level of attention as other pollutants. To understand if Cr is capable of inducing microsatellite instability (MSI) and thus compromise the DNA repair machinery, *Pisum sativum* plants were grown on soil and watered for 28 days, with different concentrations of Cr containing solutions, ranging from 20 to 2000 mg l^{-1} Cr. Roots and leaves of control and Cr-exposed plants collected and 10 microsatellites (SSR) were used to assess if Cr could cause MSI. Results indicate that up to 1000 mg l^{-1}, evidence of MSI in either roots or leaves could not be observed. However, roots of plants exposed to 2000 mg l^{-1}, displayed signs of MSI in two of the loci analyzed, corresponding to a mutation rate of 8.3%. In conclusion, we demonstrate for the first time in plants, that Cr can provoke MSI and more importantly the findings reported here give a better insight into this metal's mechanism of genotoxicity.

Keywords: chromium; genotoxicity; microsatellite; microsatellite instability, *Pisum sativum*.

Introduction

Genotoxicity is one of the most important and dramatic type of toxicity that metal can generate, not only due to the immediate threat to the organism but also because plants do not have a predetermined germ line and so, damage to somatic cells might be transferred to the gametes (Golubov *et al.*, 2010). In general, metal genotoxicity is primarily originated through the indirect action of these pollutants, either through generation of reactive oxygen species and associated oxidative stress damage or by the inhibition of the DNA repair systems, causing genetic instability and mutation accumulation (Beyersmann and Hartwig, 2008). However, some metals can interact directly with DNA and cause damage by themselves, being Cr one of the most notable cases. Cr(VI) is a known human carcinogen, which after intracellular reduction to Cr(III) can form Cr-DNA adducts (Peterson-Roth *et al.*, 2005). Salnikow and Zhitkovich (2008) discussed that apparently, two classes of Cr-DNA phosphate adducts existed: the majority (about 90%) being non-mutagenic monofunctional Cr-phosphate complexes, while the minority are mutagenic microchelates involving a phosphate group and the N^7 position of G. Later on, O'Brien *et al.* (2009) demonstrated in yeast that Cr(VI)-induced base substitution mutations accounted for at least 83.9% of the mutations observed, while deletions and insertions only corresponded to 16.1 %. Although these reports provide important information and demonstrate that Cr can be highly mutagenic, in plants the data about Cr genotoxicity is scarce and mostly focused on micronucleus formation and chromosomal aberrations. In a recent study, Rodriguez *et al.* (2011) demonstrated that Cr(VI) induced DNA degradation, clastogenicity and cell cycle arrest at the G_2/M checkpoint, which is known to be activated in order to provide cells time to repair DNA damage prior to mitosis (O'Connell and Cimprich, 2005, Culligan *et al.*, 2004). Furthermore, Labra *et al.* (2003) observed that *A. thaliana* treated with Cr(VI) presented more polymorphic AFLP bands than control plants, indicating that this metal is mutagenic for plants. With the development and improve of molecular markers, these technique have become an important tool in the evaluation of pollutant-induced mutations (*e.g*. deletions, insertions, point mutations). Microsatellites, which are short, tandemly repeated DNA sequences comprising 2–6 bp per repeating (Leonard *et al.*, 2003, Azaiez *et al.*, 2006), are one of the most commonly used molecular markers mainly due to their abundance (widespread through eukaryotic genomes), random occurrence and high degree of polymorphism (Burstin *et al.*, 2001). In humans and animals, microsatellite instability (MSI) is commonly used to detect mutations due to malfunction of the DNA mismatch repair machinery (Hirose *et al.*, 2002, Ribic *et al.*, 2003, Peterson-Roth *et al.*, 2005). In plants, despite less popular than for other organisms, some studies of MSI have been undertaken (Depeiges *et al.*, 2005, Monteiro *et al.*, 2009, Golubov *et al.*, 2010), demonstrating that this marker is suitable for evaluating mutagenesis in plants. The objective of the present investigation was to answer if Cr induced MSI instability in plants and to evaluate the suitability of this technique as genotoxicity marker.

Material and Methods

Plant conditions and Cr treatment

Pea seeds (*Pisum sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sawed in pots containing a peat:perlite mixture (4:1). Plants were grown during 28 days at 24ºC ± 1ºC, under light intensity of 200 µmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark).

Cr treatment consisted in watering plants (at least 25 per condition) during the aforementioned period of 28 days, twice per week, with 100 ml of a 1:10 Hoagland's solution containing the following concentrations of $K_2Cr_2O_7$: 0; 20; 200; 1000 and 2000 mg l^{-1} (Designated as Cr(VI) treatment). Afterwards plants were collected, rinsed thoroughly to remove substrate adhered to roots and tissues were sampled for analysis. At least two independent experiments were performed for each parameter.

Chromium analysis

The amount of Cr accumulated by control and Cr-treated tissues (at least 3 plants per condition, with 3 replicates per sample) was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France) as described by Rodriguez *et al*., (2011). Accumulation of Cr was determined in leaves and roots dried to constant weight at 60ºC. Dried tissues were placed in a crucible at 530 ºC during 14h in order to reduce samples to ashes. Then, ashes were digested in three steps by successively adding HCl dilutions (decreasing concentration in each step) and heating. After this, samples were filtered by vacuum suction using Whatman paper filter nº1 and a Buchner funnel. The crucible and then the funnel were washed with 10% HCl (v/v) to minimize loss of sample. Finally, ultra pure water was added until final volume of 14 ml was obtained and samples were analyzed by ICP-AES.

Microsatellite analysis

Total DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN,Germany) from roots and leaves (100 mg each) of control and Cr-treated plants (3 individuals per condition from 2 independent experiments), following manufacturer's instructions.

Ten SSR were chosen from the ones reported by Burstin *et al.* (2001) to represent different repetitive motifs, size, location in the genome and number of repeats. The primers were synthesized by Invitrogen (UK). From the ten SSR chosen, 2 could not be amplified (even with different PCR conditions) and thus the remaining 8 (which amplified) were used to evaluate MSI. The primers' sequence, repetitive motif and temperature are given in table 1. In order to choose the proper PCR condition, a pre-assay was performed, with the annealing temperatures ranging from 54 to 63 ºC. The optimal temperatures for each SSR are given in table 1. The remaining PCR

conditions were as instructed by the PCR's kit manufacturer (TAQ polymerase Core kit, Qiagen): each assay contained 30 ng of template DNA, in 20 pM of each primer, 0.2 mM dNTP, 1*x* Taq buffer containing 1.5 mM $MgCl_2$, 1 U Taq polymerase, in a total volume of 25 µl.

Table 1 Description of the Microsatellite used. Locus, accession number, forward and reverse primers sequence and motif were taken from Burstin et al 2001. Tm- annealing temperature used.

SSR	Locus	Accession Nº	Forward primer	Reverse primer	Motif	Tm (ºC)
1	PEAATPSYND	M94558	CTCCAGCCCAATAGTCGAAG	TCACAACCGAAGTCACAACC	$(AC)_6$	61.5
2	PEACPLHPPS	L19651	GTGGCTGATCCTGTCAACAA	CAACAACCAAGAGCAAAGAAAA	$(AT)_6$	61.5
3	PSRBCS3C	X04334	CCCAGTGAAGAAGGTCAACA	CAATGGTGGCAAATAGGAAA	$(AT)_6$	57.5
4	PSARGDECA	Z37540	CTGTTCCTCTTTCAAGCACTCC	GGGAAAGCAAAGCATGCGGATC	$(TC)_7$	61.5
5	PEARHOGTPP	L19093	ACGCTTCAACGGCAAAAT	AGGACCCCAATCACTCTCAC	$(TC)_5$	57.5
6	PSGSR1	X04763	TGAAACCACCATTCTCTGGA	AAGACCCCACTTGAAAATTACTTC	$(ATT)_5$	55.9
7	PSAJ3318	AJ223318	CAGTGGTGACAGCAGGGCCAAG	CCTACATGGTGTACGTAGACAC	$(CAT)_6$	61.5
8	PSBT2AGEN	X96764	GCAGCAGAGCTTGTCTTTGAG	GGAATCAGAAACAGCCTTGGG	$(CCT)_5$	57.5

PCR was performed in a MyiQ2 (Biorad, CA, USA) and included 1 step of 3 min at 94 ºC followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at the required Tm (Table 1) and

45 s elongation at 72°C. The final step was a 5 min elongation at 72°C. PCR products were electrophoresed in 2% agarose gels stained with ethidium bromide and running in 1*x* TBE buffer. The bands were visualized on a UV transilluminator (G:Box Syngene, Cambridge, UK). The respective software (GeneSnap) was used for image acquisition and analysis. PCR products were further evaluated by Capillary Electrophoresis (CE) on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, USA). Samples were prepared according to the modified protocol from Life Technologies Corporation: 1µL of PCR product was mixed with 25 µl deionized formamide (HI-Di™ Formamide, Applied Biosystems) and 1.0 µL of internal size-standard labeled with ROX™ (GeneScan™ 500 ROX™ Size Standard, Applied Biosystems).

Results

Cr accumulation

The amount of total Cr accumulated in control tissues was 19.72 ± 1.42 µg Cr g^{-1} DM for leaves and 24.0 ± 17.61 µg Cr g^{-1} DM for roots. Roots accumulated more Cr than shoots, being that total Cr accumulation was directly correlated to the Cr dosage used. The ratios of Cr accumulation, in respect to control, are presented in Table 2, being that all the values are significantly different from control ($P < 0.005$). The maximum difference in Cr accumulation to the control was observed in plants exposed to 2000 mg l^{-1}; leaves accumulated 27 fold more Cr than control while for roots the difference in accumulation was 51 fold.

Table 2 Cr accumulation in shoots and roots of plants exposed to the given concentration of Cr. For each treatment and tissue, the value given is the mean [Cr treatment]/[Cr control tissue] ± standard deviation.

Cr [mg l^{-1}]	Leaves (µg g^{-1} DM)	Roots (µg g^{-1}DM)
20	6.4 ± 2.73^{b}	12.3 ± 4.50^{b}
200	7.2 ± 1.36^{b}	22.4 ± 4.47^{c}
1000	18.2 ± 3.94^{c}	30.5 ± 7.70^{d}
2000	27.5 ± 3.57^{d}	51.5 ± 5.22^{e}

Microsatellite analysis

The Controls and Cr-treated plants PCR products fragment sizes, as well as the sizes predicted from the information in the BLAST database are presented in table 3. The comparison between the control's and the predicted PCR product size revealed that the highest difference observed was of 2 bp, except for SSR 5 that presented a difference of 115 bp. We did not found evidence of Cr-induced MSI in leaves, as there were no differences in allele size between control and exposed leaves. For roots, on the other hand, the highest Cr dosage induced MSI in SSR 2 and SSR6, with difference to the respective controls of 6 bp (Figure 1) and 3 bp (Figure 2). These

results are evidence of Cr-induced MSI in roots, presenting a mutation frequency of 8.3% ((1 root plant/3 plants analyzed)×(2 SSR/8 SSR analyzed)).

Table 3 Allele size of the SSRs used to evaluate Cr-induced MSI. For each SSR, the values (in bp) presented are: Allele's predicted size, using the information on the Blast database; the control tissues' allele size; exposed leaves allele size and exposed roots allele size. Because difference among Cr treatments were not observed (except for 2 cases), the allele size of the samples of the same tissue were displayed in the same column (either leaves or roots).

SSR	Predicted Size (BP)	Size Control (bp)	Exposed Leaves Size (bp)	Exposed Roots Size (bp)
1	208	208	208	208
2	126	125	125	119*
3	210	206	206	206
4	258	255	255	255
5	195	315	314	316
6	196	198	198	198; 201**
7	166	168	168	168
8	269	267	267	267

*One sample of a Cr-treated (2000 mg l^{-1}) root showed an allele of 119 bp

** One sample of a Cr-treated (2000 mg l^{-1}) root showed an allele of 201 bp

Discussion

The evaluation of genotoxicity markers, which are highly sensitivity and have an impressive informative value, has been underexplored in phytotoxicity studies, even though there is a relative abundance of assays using micronuclei and cytogenetic techniques to detect DNA damage. The development of PCR based techniques allowed to apply a molecular approach in the study of genotoxicity, presenting new data and unveiling part of the mechanism by which pollutants can affect plants. For metal toxicity in particular, studies like the ones of Kovalchuk *et al.* (2000), Labra *et al.* (2003), Monteiro *et al.*(2009) demonstrated the usefulness of these markers.

Our results demonstrated that MSI was not observed in plants (roots or leaves) exposed to Cr, for doses up to 1000 mg l^{-1}. This does not mean that DNA damage and mutations could not have occurred, specially taking into consideration the data presented by Rodriguez *et al.* (2011), in which by the Comet assay and flow cytometry detected significant levels of DNA damage and clastogenicity in plants exposed to the same conditions that we report now. It is possible that up to those dosages, and specially in roots which accumulated significantly more Cr than leaves of plants exposed to the same Cr dosage, the DNA repair machinery managed to overcome putative damage that might have been caused by Cr exposure. Like with our data, Labra and co-workers

(2004) did not found changes in the DNA patter at the lower dosages; only at 20 mg l^{-1} of Cr were the first changes observed.

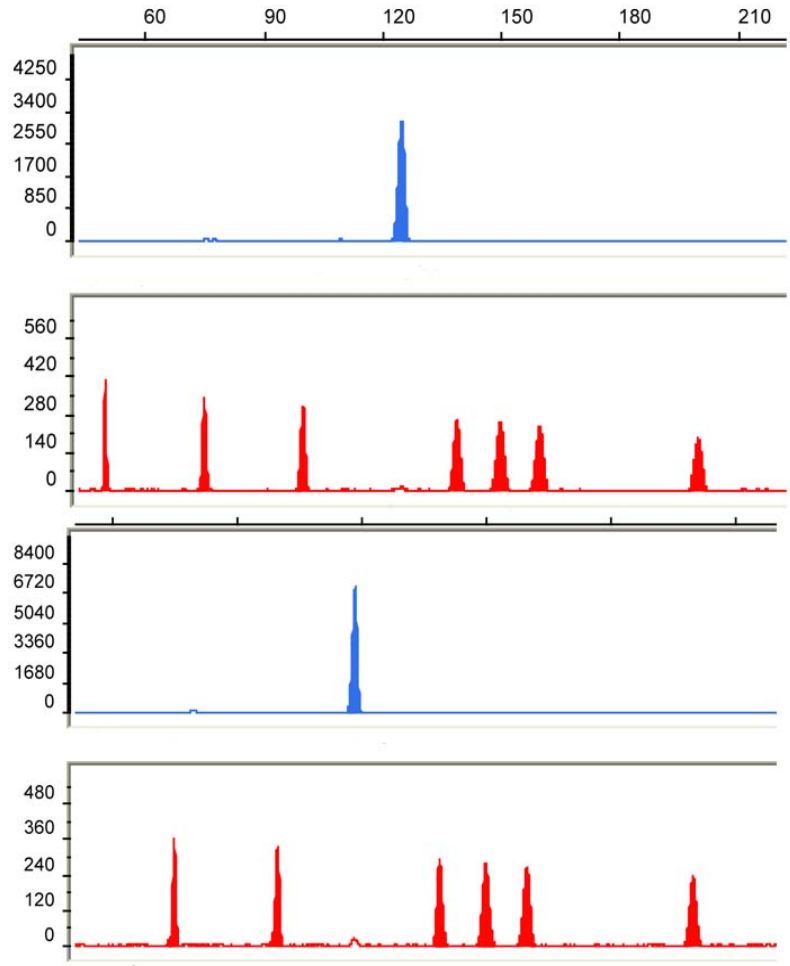

Figure 1 Electrophoretograms of SSR 2. The Electrophoretrogram correspond to roots from control and Cr-treated plants (2000 mg l^{-1}). Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity. Top to bottom: Control root (blue peak); ROX 500 (Internal control- red peaks); Exposed root (blue peak), ROX 500 (internal control- read peaks). Samples were run simultaneously with each internal control.

It could be discussed that those authors found evidence of toxicity in such low dosages while compared to our results, but a couple of reasons might explain the difference between both reports. Besides the more than probable differences in uptake and tolerance to Cr toxicity between rapeseed and pea plants, the bioavailability of Cr in our study should be significantly lower than in the one of Labra *et al.* (2004) as our plants were grown on soil matrix while they exposed their plants directly to Cr-containing solution, which is more readily uptake by plants and accumulated in higher concentrations (even though those authors did not present data on Cr accumulation to allow a comparison).

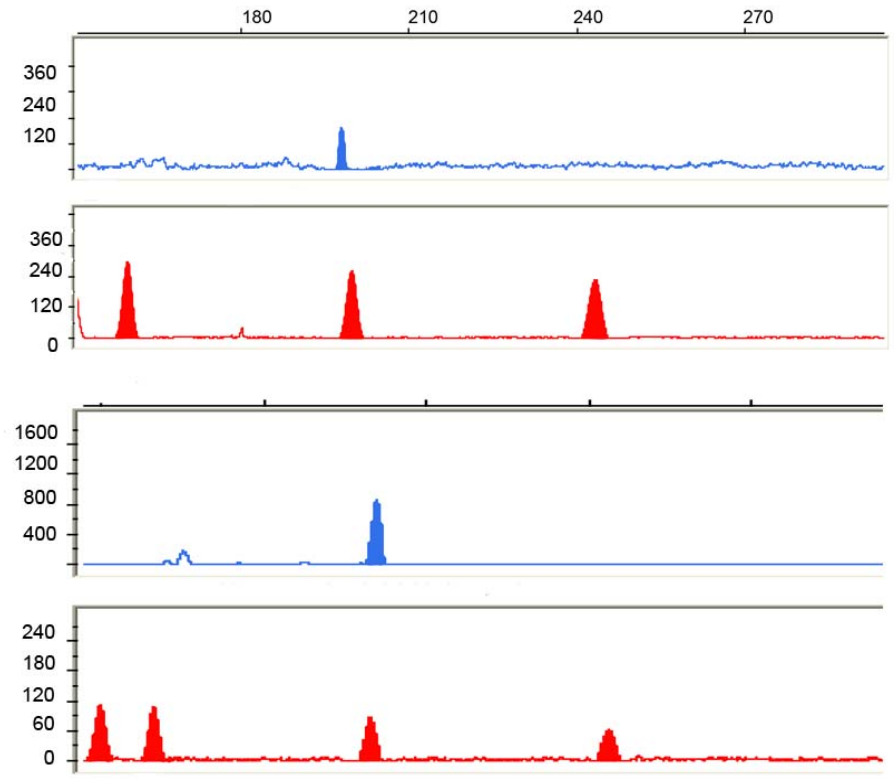

Figure 2 Electrophoretograms of SSR 6. The Electrophoretrogram correspond to roots from control and Cr-treated plants (2000 mg l^{-1}). Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity. Top to bottom: Control root (blue peak); ROX 500 (Internal control-red peaks); Exposed root (blue peak), ROX 500 (internal control)

Unlike all other conditions tested, we were able to detect, in roots exposed to 2000 mg l^{-1} of Cr, evidence of Cr-induced MSI, in two of the SSR evaluated. SSR2 (inserted in the locus for *Pisum sativum* chloroplast photosystem I 24 kDa light harvesting protein) from Cr-treated roots presented an allele of 119 bp instead of the 125 bp observed in control while SSR6 (*Pisum sativum's* gluthamine synthase locus) from treated roots presented an allele with 3 bp more than that of the control (198 bp); these results are indicative of Cr-induced MSI, at a mutation frequency of 8.3%. Monteiro *et al.* (2009), like in our case, only observed MSI in lettuce roots exposed to the highest dosage tested in that work but the mutation frequency observed was lower (3.7 %). The difference between both mutation frequencies could be explained by several factors like differences between species tolerance to metal toxicity; dosage used and bioavailability of the metals; Cr and Cd having different mechanism of toxicity and targets. As a matter of fact, while Rodriguez *et al.* (2011) detected Cr-induced G_2/M cell cycle arrest and polyploidization in roots exposed to the same conditions of our assay; Monteiro *et al.* (2010) in an assay with similar experimental conditions to those used in Monteiro *et al.* (2009) did not detect cell cycle arrest or DNA content changes, which might support the hypothesis of Cr and Cd having different mechanism for

genotoxicity induction. Moreover, the results presented here might also explain why Rodriguez *et al.* (2011) only detected polyploidization and cell cycle arrest in roots of plants exposed to the 2000 mg l^{-1}, despite evidence of significant DNA damage in lower doses. It is possible that the DNA repair mechanism is only significantly compromised in roots exposed to the maximum dosage, resulting in mutations, arrest at the G_2/M checkpoint and polyploidization.

In conclusion, we report for the first time, evidence of Cr-induced MSI in plants. Furthermore, besides demonstrating the suitability of SSRs as a marker of metal phytotoxicity, we also present important data that could help to explain Cr-induced genotoxicity.

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CHAPTER III

PHOTOSYNTHETIC STATUS OF *P.SATIVUM* EXPOSED TO ABIOTIC STRESSES

CHAPTER III-1

CHLOROPLAST FUNCTIONALITY ASSESSMENT BY FLOW CYTOMETRY: CASE STUDY WITH PEA PLANTS UNDER PARAQUAT STRESS

Chapter accepted for publication as an original article in a SCI journal:

Rodriguez E, Azevedo A, Costa A, Serôdio J and Santos C (2011) Chloroplast functionality assessment by flow cytometry: Case study with Pea plants under Paraquat stress. Photosynthetica (accepted with minor revision).

Abstract:

Photosynthesis is one of the most important processes in plant biology and in the development of new methodologies that allow a better understanding and characterization of the photosynthetic status of organisms, which is invaluable. Flow cytometry (FCM) is an excellent tool for measuring fluorescence and physical proprieties of particles but it has seldom been used in photosynthetic studies and thus the full extent of its potentialities, in this field of research, remains unknown**.** To determine the suitability of FCM in photosynthesis studies, pea plants were exposed to Paraquat and their status was analyzed during 24h. FCM was used to evaluate the integrity (volume and internal complexity) and the relative fluorescence intensity (FL) of chloroplasts extracted from those plants. To elucidate which type of information the FL conveys, FL values were correlated with the minimum fluorescence level (F_0), maximum fluorescence level (F_m) and maximum photochemical efficiency of PSII (F_v/F_m), obtained by using Pulse-Amplitude-Modulation (PAM) fluorometry. Results indicate that: a) the biomarkers used to evaluate the structural integrity of the chloroplasts were more sensitive to Paraquat exposure than the ones related to fluorescence; b) the variation of the chloroplast's structure, as time progressed, pointed to a swelling and subsequent burst of the chloroplast which, in turn, compromised fluorescence emission; c) FL presented a high and significant correlation with the F_v/F_m and to a lesser degree with F_m but not with F_0; d) pigment content did not reveal significant changes in response to Paraquat exposure and is in agreement with the proposed model, suggesting that the cause for fluorescence decrease is due to chloroplast disruption. In sum, FCM proved to be an outstanding technique to evaluate chloroplastidal functional and structural status and therefore it should be regarded as a valuable asset in the field of photosynthetic research.

Keywords: chlorophyll fluorescence; chloroplast; flow cytometry; paraquat; photosynthesis; pulse-amplitude modulation fluorometry.

Introduction

Among the many aspects regarding photosynthetic processes, those producing stress to plants generate major concerns. Heavy metals, organic pollutants or drought stress, to name a few, can affect diverse steps of photosynthesis (*e.g.* Azevedo *et al*., 2005, Vernay *et al*., 2007, Hattab *et al*., 2009, Váňová *et al*., 2009, Dias and Brüggemann, 2010) reducing crop yield, causing deleterious effects to ecosystems and eventually affecting human health. It is therefore important to develop new methodologies to study the photosynthetic status of organisms. These methodologies should provide new biomarkers to rapidly, easily and accurately assess plant status, facilitating the data collection process, which would be otherwise, achieved through laborious and time-consuming procedures.

Among the various techniques available to study photosynthesis, variable chlorophyll fluorescence, mainly through PAM fluorometry (Schreiber *et al*., 1986, Maxwell and Johnson, 2000) became widely used as a rapid and sensitive method to quantify *in vivo,* the performance of the photosynthetic apparatus, including evaluation of photo-inhibitory and pollutants effects (*e.g.* Marwood *et al.,* 2000, Juneau *et al*., 2002, Ali et al., 2006). Because the study of phytotoxicity at the photosynthetic level has been carried out for a long time, most of the parameters measured are well established and the techniques used for these evaluations are part of the classical approaches used in photosynthesis studies. FCM on the other hand, and despite a promising start in the 90's (Ashcroft *et al*., 1986, Xu *et al*., 1990, Schroder and Petit, 1992, Pfundel *et al*., 1996), has seldom been applied to study the functional aspects of photosynthetic processes. FCM allows measuring the physical-chemical proprieties of particles flowing in a fluid stream, combining high speed (up to thousands of events per second), accuracy and high sensitivity (distinguishing variation in the submicron range, (Dubelaar and Jonker, 2000), to the capability of performing multi-parametric assays through the simultaneous analysis of fluorescence and the light scattering proprieties of particles (Loureiro *et al*., 2006).

FCM's ability to evaluate the interaction between particle and light beam, can be used to characterize the morphology and integrity of chloroplast: light reflected in small angles (Forward Scatter -FS) is related to the particle's volume/size while light reflected in large angles (Side Scatter -SS) is related to the particle's granularity/internal organization. Schroder and Petit (1992) demonstrated that a combination of FS and SS alone could be used to discriminate between intact and disrupted chloroplasts. Morovoer, a comparison between the results obtained by FCM and the ferricyanide test (a common test used to evalute chloroplast integrity) demonstrated that both techniques provided highly similar results (80% intact chloroplast by ferricyanide test, 76% by FCM). FCM presentes several advantages over other methods used to evaluate chloroplast integrety (*e.g.* ferricyanide test, phase-contrast microscopy, gluconate-6-phosphate dehydrogenase activity), namely, as it can be used to evaluate thousands of chloroplast per second with great accuracy while simultaniously analyzing other parameters, whether it be autofluorescence or the fluorescence emitted by a labelled target. Furthermore, due to the high sensitivity of FCM, subpopulations can be detected and special types of cytometers called fluorescence-activated cell

sorters can be used to separate, purify and recover populations of interest for further analyses. In what concerns chloroplast autofluorescence, Xu *et al.* (1990) theorized that a 488 nm flow cytometer laser is able to detect the Chl *a* fluorescence emitted when the PSII reaction centers are locked in the Q_A^- state, that is, maximum fluorescence, opening the possibility of using this technique to study functional aspects of the photosynthetic apparatus.

The objective of the investigation presented here was to evaluate the applicability of FCM in phytotoxicity studies, by comparing FCM and PAM measurements, in order to understand if the parameters provided by FCM can be used as biomarkers of photosynthetic impairment. To achieve this, Paraquat, an herbicide known to affect photosynthesis (Bromilow, 2004), was used as a model stress factor.

Material and methods

Plant growth and experimental conditions

Pisum sativum L. cv Telephone seeds were germinated and grown in a peat/perlite mixture (4:1) for three weeks, at a temperature of 24 ºC ± 1 ºC, under a light intensity of 200 µmol m^{-2} s^{-1} and photoperiod of 16 h/8 h (light/dark). After this, were sprayed with a photosynthesis acting herbicide containing Paraquat as the active agent at the maximum permitted concentration for field spraying (1% W/V). Plants were analyzed at 3 h, 6 h, 9h, 12 h, 15 h, 18 h, 21 h and 24 h of exposure to the herbicide, and were adapted to the dark for 30 min prior to isolation/analysis.

Results presented are the outcome of 3 independent experiments.

Chloroplast isolation and Flow cytometric analysis

For FCM analysis, chloroplast isolation was performed using the protocol developed by Robinson and Mant (2002) with some modifications (4,000 × *g* centrifugation times were increase from 1 to 2 min and nylon mesh was used to filter the suspension instead of miracloth). In brief, leaves were harvested as needed and grinded with a mortar and pistil (automated devices can be utilized instead), in Hepes-Sorbitol buffer (HS), containing sorbitol and Hepes. The suspension was filtered through two 50 µm nylon meshes, and placed in a swing out rotor centrifuge for 2 min at 4,000 × *g*. Supernatant was collected and placed in 2 ml of a 35% percoll solution, and centrifuged for 8 minutes at 1,400 × *g*. Pellet was collected, re-suspended and centrifuged twice in HS buffer at 4,000 × *g* for 2 min, to wash the Percoll ® away and then analyzed in a flow cytometer (*Coulter Epics XL, Beckman Coulter*, Hialey, FL, USA). The FS and SS of each particle was registered; the autofluorescence (as relative fluorescence intensity- FL) emitted by chloroplasts was collected using a Argon ion laser operating at 488 nm, a 645 nm dichroic long pass filter and a 675 nm band pass filter. Three individuals were analyzed per condition and three replicates were sampled by individual. From the analysis of the level of FL emitted and the FS and SS values of control chloroplasts, two populations were defined, A and B. Population B comprises particles with high

FL and well define scatter values (intact chloroplast); population A includes particles with low FL emission and diffuse scatter values, lower than those of Pop B (damaged chloroplast). This classification was validated by comparing this data with the one resulting from the analysis of chloroplasts damaged by osmotic stress (incubated 30 min in a solution containing 1% v/v Triton X-100 and 100 mM of NaCl pH 7.5). With these parameters set, the voltage of the cytometer and the regions characterizing these populations were kept constant for the remaining of the assay.

PAM fluorometry

Variable chlorophyll fluorescence was measured, in intact leaves, using a portable PAM fluorometer (*Portable Junior-PAM*, *Gademann Instruments* GmbH, Germany). This instrument applied a modulated blue light (LED-lamp peaking at 470 nm, half-bandwidth of 31 nm) as source for measuring, actinic and saturating light, emitted at a frequency of 25 Hz when measuring the minimum fluorescence level (F_0) or 1.2 kHz when measuring other fluorescence parameters. Fluorescence was measured in situ using a 1.5 mm-diameter plastic fibreoptics (*Edmund Optics*, UK), maintained at a constant distance of 2 mm from the leave surface. After a 15 min period of dark adaptation, one saturating pulse of 0.8 s was applied to measure the minimum and maximum fluorescence levels, F_0 and F_m and to calculate the F_v/F_m.

Pigment content

Chl *a* and Chl *b* were quantified, spectrophotometrically, following the protocol developed by Sims and Gamon (2002). After grinding in an acetone:Tris (80:20 v/v) buffer solution, the suspension was vortexed and centrifuged at 2,800 × *g* for 5 min. The supernatant was stored in ice and covered with aluminium foil and the pellet was treated as above. The new supernatant was added to the previous one, and then the sample was diluted to a final volume of 6 ml. The absorbance of 4 individuals per condition (3 replicates per individual) was collected in the following wavelengths 537 nm, 647 nm and 663 nm, which then were used to calculate Chl *a* and *b* concentration with the following formulas:

$$\mathbf{Chl_a} = 0{,}01373\ A_{663} - 0{,}000897\ A_{537} - 0{,}003046\ A_{647}\ ;$$

$$\mathbf{Chl_b} = 0{,}02405\ A_{647} - 0{,}004305\ A_{537} - 0{,}005507\ A_{663}$$

Statistical analysis

Data collected was analyzed with a one-way ANOVA, using SPSS 17. Whenever significant differences ($P \leq 0.05$) were detected, a Holm-Sidak multiple-comparison test was performed. Due to the similarity between the controls of each hour of exposure, for display and statistical purposes, these were considered as a single control condition for all the analyses. FCM

and PAM flourometry data were correlated by Pearson's correlation using SigmaPlot for Windows ver 11.0.

Results

Physical characterization

Paraquat exposure caused severe effects in the green parts of the plants, especially after 12 h of exposure (Figure 1 *C* and *D*), with progressive wilting, loss of brightness and graying of the leaves, culminating with the death of the aerial part of the plant in about 48 to 72 h of exposure.

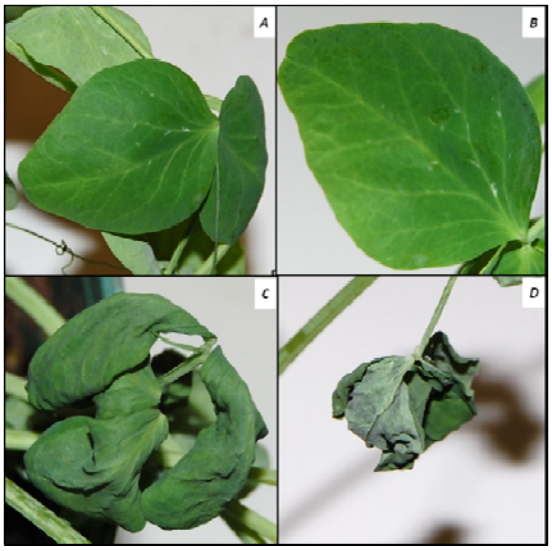

Figure 1 Leaves of *Pisum sativum* exposed to Paraquat. *A*- control, *B*- 12 h of exposure, *C*- 15 h of exposure, *D*- 24 h of exposure.

Flow cytometric analysis

Results from the analysis of intact and damaged chloroplast (osmotic stress) are presented in Figure 2. The FS *vs* SS cytogram of intact chloroplast (Figure 2*A*) shows a well defined population of chloroplasts; on the other hand, broken chloroplasts (Figure 2*B*) have heterogeneous distribution and lower scatter values. When comparing the aspect of intact and damaged chloroplast (Figure 2*C* and *D*, respectively) it is easily observable that osmotic shock leads to chloroplast burst, eventually disintegrating it into smaller fragments. The overlay of intact (Figure 2, gray) and broken (Figure 2, white) chloroplast's FL histogram demonstrates that the main population of intact chloroplast emits higher FL than broken/damaged ones. Within the controls, a small % of the chloroplast emits lower FL, overlapping with the histogram of damaged chloroplast. This subpopulation also had lower scatter values than those of high FL emitting chloroplast, presenting an overall profile alike damaged chloroplast, and was thus defined as Pop A.

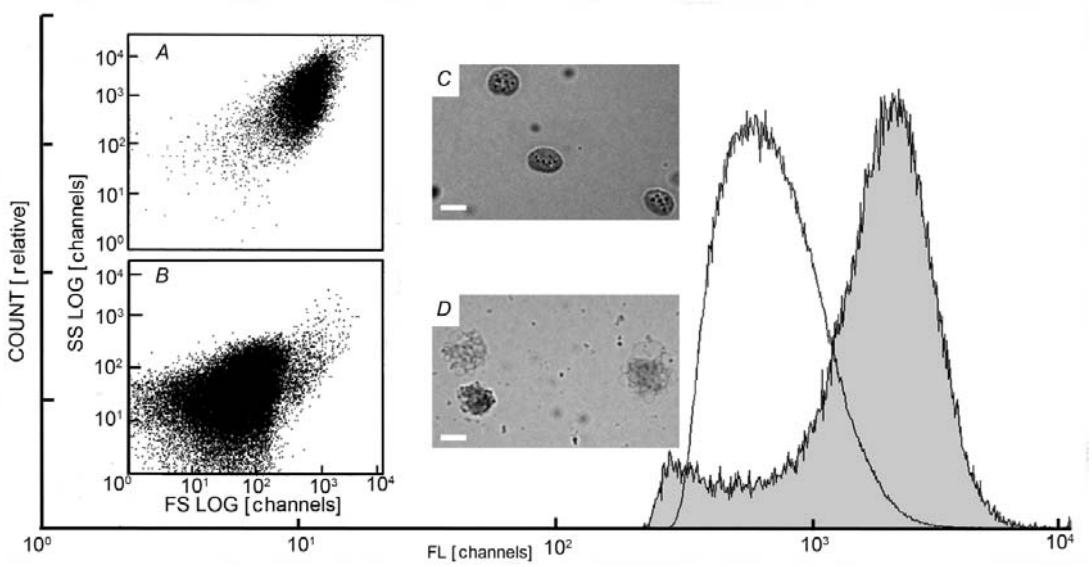

Figure 2: Analysis of intact and broken/disrupted chloroplast. The relative fluorescence intesintity (FL) histogram presents an overlay of intact (grey) and osmotically shocked chloroplast (white). Inserted in the figure are the FS vs SS cytograms of isolated (*A*) and osmotically shocked chlorplasts (*B*) as well as the pictures of those chloroplast taken with a 40x magnification (*C*- intact, *D*- osmotically shocked).

Exposure to Paraquat caused significant changes in the volume and granularity of chloroplast, when compared to controls (Figure 3*A*). For Pop A, after some heterogeneous variation until 9 h of exposure, a steady and significant increase was observed in the subsequent hours, peaking at 24 h of exposure (Figure 4*A*).

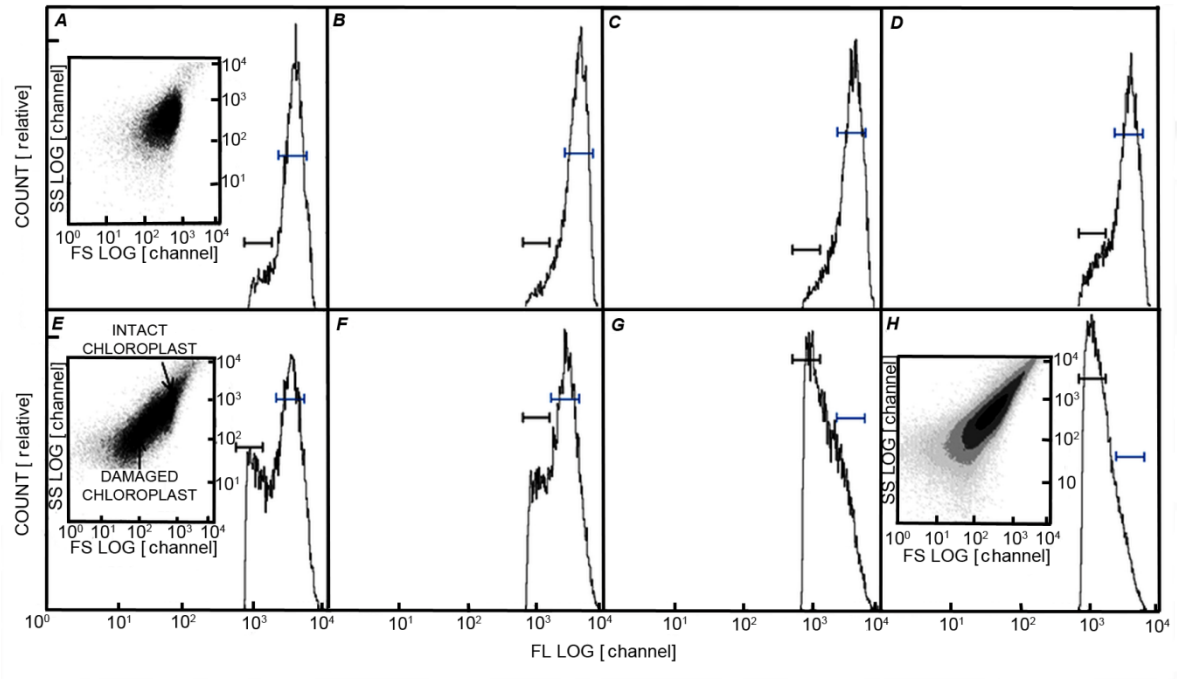

Figure 3: FL Histograms of chloroplast isolated form plants exposed to Paraquat. *A*- control, *B*- 3 h of exposure, *C*- 6 h of exposure, *D*- 9 h of exposure, *E*- 12 h of exposure, *F*- 15 h exposure, *G*- 18 h exposure, *H*- 24 h exposure (The histogram at 21 h of exposure was omitted due to its similarity with the one at 24h). In each hystogram 2 regions are marked, the blue region defines Pop. B and the black one defines Pop. A. Inserted in histograms *A*, *E* and *H*, are the respective cytograms of FS vs SS in logarithmic scale (*n* = 9).

The volume and granularity values of Pop B presented different responses during the first 3 times of analysis after exposure to Paraquat (3 h, 6 h and 9 h); if volume decreased from 0 to 9 h of exposure (45 % decrease), granularity increased from 0 to 3 h, and then decreased until 9 h of exposure (Figure 4*B*). After 9 h of exposure, both parameters presented the same variations: a sharp increase from 9 h to 12 h (around 55%) and then a high decrease from 15 h to 21 h (60% for FS, 50% for SS, lowest values observed).

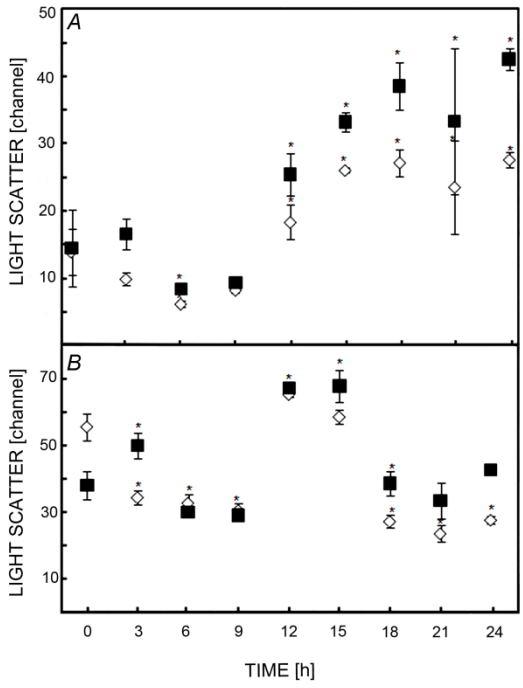

Figure 4: Volume (FS- diamond) and granularity (SS- closed squares) variation of chloroplasts isolated from plant exposed to Paraquat for the given time. Values given are the mean and standard deviation (as error bars) of Pop. A (*A*) and Pop. B. (*B*) Values marked with a (*) are significantly different from control ($P \leq 0.05$) (*n* = 9).

Nonetheless, the decrease was more pronounced in volume, being that the last 3 analysis points (18, 21 and 24 h) were significantly lower ($P \leq 0.05$) than the control, while for the granularity, the decrease for this time gap, levelled the values with the control ones. The maximum variation observed for FS was between 12 h and 21 h (a decrease of 64%; 58% between control and 21 h)

while for SS, the highest variation was observed between 9 h and 15 h (increase of 58%; 44% between control and 15 h).

The mean and median FL for populations A and B were collected and compared but there were not significant differences between the mean and median FL ($P > 0.05$), within any of the populations.

Pop A showed little variation in FL ($P > 0.05$) throughout the experiment (Table 1, Figure. 3*F*, *G* and *H*), with the largest variation being around 10% (between 6 and 18 h of exposure).

Table 1 Fluorescence emission of chloroplast extracted from plants exposed to Paraquat for the given time (h). Parameters presented are: Fluorescence intensity (FL), of intact chloroplast (Pop B); broken/damaged chloroplast (Pop A) and both populations (Pop A+B). Values are given as mean/median ± standard deviation for each population. Values followed by different letters are statistically different ($P \leq 0.05$).

T [h]	FL log [Channels]					
	Pop. B		Pop. A		Pop. A + B	
	Median	Mean	Median	Mean	Median	Mean
0	361.4±8.49^{a}	364.3±11.87^{a}	98.8±1.01^{a}	97.3±1.21^{a}	300.1±11.73^{a}	340.5±15.34^{a}
3	406.4±9.42^{b}	385.2±1.22^{b}	101.6±2.60^{a}	100.2±1.05^{a}	265.1±7.91^{a}	313.9±2.03^{a}
6	458.5±26.59^{b}	428.3±55.65^{b}	107.0±4.46^{a}	105.8±4.11^{a}	209.4±28.14^{b}	202.4±40.45^{b}
9	333.5±7.67^{ac}	314.0±1.21^{ac}	101.0±1.37^{a}	99.2±1.99^{a}	258.0±3.87^{ab}	309.4±5.29^{ab}
12	322.1±15.35^{c}	316.9±7.62^{c}	100.4±4.12^{a}	98.9±3.58^{a}	233.6±2.38^{b}	258.8±3.87^{b}
15	278.8±3.64^{d}	281.5±1.05^{d}	100.8±2.69^{a}	99.6±1.10^{a}	218.8±1.42^{b}	234.3±1.25^{b}
18	238.2±5.37^{d}	249.6±0.95^{d}	95.7±3.73^{a}	93.3±4.63^{a}	166.6±2.87^{c}	153.0±1.04^{c}
21	200.5±1.86^{e}	211.1±7.35^{e}	99.6±5.49^{a}	99.7±9.96^{a}	147.8±3.63^{c}	135.4±1.08^{c}
24	208.2±1.32^{e}	224.3±1.07^{e}	105.0±4.76^{a}	103.2±3.99^{a}	149.6±16.06^{c}	142.4±6.38^{c}

Pop B's FL suffered significant variation as time progressed: significant increase was observed from 0 to 6 h of exposure ($P \leq 0.05$) followed by a decrease to control levels at 9h of exposure ($P > 0.05$). From 12 h of exposure onwards, FL values are significantly lower than control ($P \leq 0.05$), with a sharp decrease between 12 and 15 h, followed by a less pronounced decrease until 21 h, where the lowest value was observed (40% lower than control).

The % of intact chloroplasts (Pop B) suffered little variation within the first 12 h of exposure to Paraquat ($P > 0.05$). After this, a constant decrease in the percentage of intact chloroplast was observed, being the lowest value (19%) observed at 24 h of exposure (Table 3). The % of chloroplasts in Pop A increased with time of exposure reaching a maximum of 81% at 24 h after exposure.

Table 3 Percentage of intact (Pop B) and damaged/broken chloroplast (Pop A.) along time of exposure to Paraquat. Values are given as mean percentage and standard deviation of the mean. In each column, values followed by the same letter are not statistically significant ($P > 0.05$).

Exposure time [h]	Pop B [%]	Pop A [%]
0	75 ± 6.4 ^{ab}	25 ± 2.1^{ab}
3	78 ± 5.6^{ab}	22 ± 6.2^{ab}
6	83 ± 5.7^{ab}	17 ± 10.1^{ab}
9	87 ± 6.7^{a}	13 ± 3.2^{a}
12	64 ± 4.5^{b}	36 ± 6.9^{b}
15	77 ± 5.5^{ab}	23 ± 4.1^{ab}
18	32 ± 3.2^{c}	68 ± 9.0^{c}
21	28 ± 2.8^{c}	72 ± 7.2^{c}
24	19 ± 1.8^{c}	81 ± 6.8^{c}

Photosynthetic yield analysis

The variation of F_v/F_m, F_m and F_0 along the time of exposure is presented in Figure 5. F_v/F_m decreased markedly throughout the experiment; the values measured after the first 12 h of exposure were significantly higher than those recorded during the last 12 h. The lowest sets of values were observed after 24 h of exposure to Paraquat, which were significantly lower ($P < 0.05$) than all other values. The F_0 presented little variation throughout the duration of the experiment ($P > 0.05$), being that the maximum variation, in respect to control, was of 23% (12 h exposure). Regarding F_m, after 3 h of exposure this parameter had already suffered a significant plunge of 40% in respect to the control ($P \leq 0.05$). After a slight increase at 6 h, F_m steadily decreased until 15 h of exposure (74% lower than at 0 h) and then little variation ($P > 0.05$) was seen until 24 h of exposure (77% lower than control).

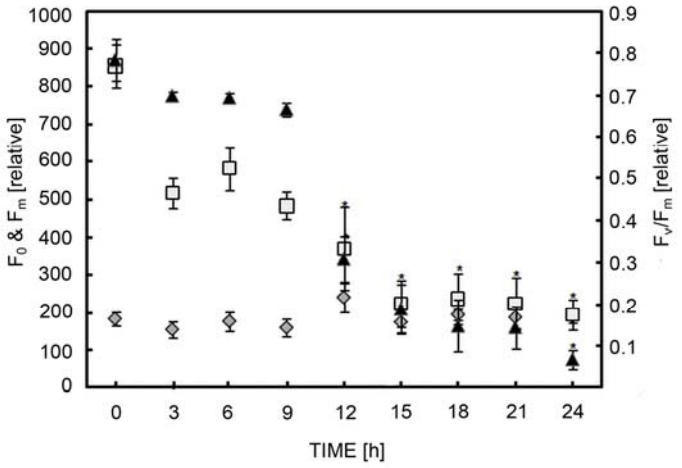

Figure 5: Variation in F_v/F_m (right axis, closed triangle), F_0 (diamond) and F_m (left axis, open square) of pea plants after exposure to Paraquat. Values are presented as the mean plus error bar (SD of the mean) ($n = 10$).

Correlation between FCM and PAM parameters

The relationship among the endpoints evaluated by FCM and PAM fluorometry was analyzed using a Pearson's correlation and is displayed in Figure 6 and Table 3. Also and in order to better correlate FCM data with PAM fluorometry, in addition to Pop A and Pop B, a third population was also considered for analysis, consisting in a combination of Pop A and Pop B (Pop A + B). The FL of Pop B (mean or median) and the F_v/F_m presented the best correlation of all the parameters assayed (r=0.883 and P=0.0016), followed by the FL of Pop A+B and the F_v/F_m (mean FL r=0.810, P= 0.004; median FL r=0.842, P=0.008). The F_m ($P \leq 0.02$) also had a positive correlation with the FL of Pop B and Pop A + B but to an inferior degree of that observed for the F_v/F_m. The F_0 did not present significant correlation with any of the parameters assayed; in fact, the correlations coefficients were all negative. Likewise, Pop A did not present significant relationship with F_v/F_m, F_0 or F_m ($P > 0.05$).

Pigment quantification

Pigment content was the only parameter that was not affected by Paraquat exposure as the quantification of both types of chlorophylls presented no significant variation ($P > 0.05$) throughout the experiment (Table 4).

Table 3: Pearson's correlation coefficient among the FL (mean or median) of the populations evaluated (A, B and A + B) and the F_0, F_m and F_v/F_m of plants exposed to Paraquat. Level of significance is represented by *($P \leq 0.05$), ** ($P \leq 0.001$) or n.s. ($P > 0.05$). The F_0, F_m and F_v/F_m were measured in intact leaves while the FL was measured in extracted chloroplasts.

		F_0	F_m	F_v/F_m
FL Pop A	mean	-0.202 $^{n.s.}$	0.067$^{n.s.}$	0.177$^{n.s.}$
	median	-0.205 $^{n.s.}$	0.081 $^{n.s.}$	0.196$^{n.s}$
FL Pop B	mean	-0.268 $^{n.s.}$	0.799**	0.883**
	median	-0.295 $^{n.s.}$	0.766*	0.883**
FL Pop A+B	mean	-0.274 $^{n.s.}$	0.767*	0.842**
	median	-0.244 $^{n.s.}$	0.830**	0.810**

Table 4 Pigment concentration (Chl *a* and Chl *b*) extracted from Pea plants exposed to Paraquat for the given time (h). Values are presented as mean ± standard deviation of the mean.

Time [h]	Chl a [µmol ml^{-1}]	Chl b [µmol ml^{-1}]
0	0.226±0.0369	0.111±0.0200
3	0.258±0.0238	0.113±0.0081
6	0.259±0.0248	0.131±0.0096
9	0.267±0.0409	0.132±0.0142
12	0.273±0.0148	0.123±0.0156
15	0.211±0.0292	0.093±0.0162
18	0.221±0.0430	0.100±0.0215
21	0.262±0.0413	0.111±0.0064
24	0.242±0.0343	0.120±0.0193

Discussion

The characterization of the chloroplast population allowed distinguishing between intact chloroplasts (well defined scatter values and high FL) and broken/damaged chloroplast (low FL and scatter values). The classification of the events in Pop A, as broken/damaged chloroplast is supported by the analysis done to osmotically broken chloroplasts, which presented similar profiles to those of Pop A. These observations are in agreement with the previous FCM reports (Ashcroft *et al*., 1986, Schroder and Petit, 1992), in which populations were defined based on fluorescence emission and scatter values. From this, we can conclude that the methodology adopted to isolate chloroplast used in our investigation provides a good yield of intact chloroplasts (nearly 75%) and is suitable for FCM studies.

The biomarkers assessed by FCM were severely affected by Paraquat being that the ones related to morphology were more sensitive than FL. This statement is based on the fact that significant structural modifications were observed prior to any significant variation in FL. After 3 h of exposure, intact chloroplasts decreased in volume but their internal complexity and FL significantly surpassed the ones of the control. This might be due to a rearrangement of the chloroplast's grana all together with a stimulation of the electron transporter chain of the PSII, resulting in the observed FL increase. This hypothesis is supported by the reports of Po and Ho (1997) and Neuhaus and Stitt (1989) in which small doses of Paraquat increased electron transport due to its action as a catalytic electron acceptor at the acceptor side of PSI, allowing a net transfer of electrons from water through PSII and PSI. In our case and despite the fact that a high dose was used, it is likely that the response of chloroplast to the first hours of exposure could have mimicked the effect of lower dosage and as time progressed, the effects evolved to what those authors observed for higher doses (inhibition of the process).

The progressive decrease in Pop B's FL (shift to the low FL zone) observed in Fig. 3, is in agreement with the work of Schroder and Petit (1992) that detected the same shift in the FL peak of osmotically shocked chloroplasts. We also found a high similarity between the profiles of chloroplast extracted from Paraquat-treated plants (Figure 3) and osmotically lysed ones (Figure 2*B*) suggesting that Paraquat induces osmotic-like stress. Data provided by Iturbe-Ormaetxe *et al*. (1998) supports our findings; they demonstrated that water deficit and Paraquat exposure

produced similar symptoms in pea plants. Moreover, the sudden and high increase followed by the rapid drop in volume observed between 9 and 18 h of exposure is consistent with a swelling of the chloroplast, followed by their burst. This, together with the substantial decrease of FL, indicates that the critical period of Paraquat exposure is situated between 9 and 18 h after exposure and that it is highly likely that the osmotic-like stress induced by Paraquat is responsible for the decrease in FL observed.

Leaves from control plants presented values of F_v/F_m around 0.8, which is close to the maximum values obtained for healthy plants (Krause and Weis, 1991, Maxwell and Johnson, 2000). Paraquat exposure caused a constant decrease in F_v/F_m, but this was only significant after 12 h of exposure (decrease of 50% to control) being in agreement with the observed pattern of FL decrease in FCM. Nonetheless, FCM seems to be more sensitive to small variations in fluorescence emission, detecting subtle changes that were not detected by PAM fluorometry.

Previous studies trying to elucidate what were the characteristics of FL as measured by FCM, lead to contradictory conclusions: Ashcroft *et al.* (1986) concluded that FCM measured F_0 while Xu *et al.* (1990) found evidence that FCM measured F_m. The latter demonstrated that increasing the incubation temperature caused a decrease in fluorescence intensity (like what was expected for F_m but not for F_0) and that the application of Diuron (Herbicide that inhibits photosynthetic electron transport by occupying the binding site of QB) caused little variation in fluorescence intensity (once again more like F_m than F_0). They also argued that due to the high intensity of the cytometer's light source, all the reaction centers underwent at least one turnover and the system reached the F_m state. Our results showed that FL (mean or median) correlated better with F_v/F_m than with F_m and not at all with F_0. It is worth mentioning that in the work of Xu *et al.* (1990) all the experiments were carried out in order to establish if FL presented was more similar to F_0 or F_m; the F_v/F_m was not considered. In agreement with our findings, Neale *et al.* (1989) working with in *Chroomonas* sp. found that, despite the high light intensity of a cytometer ($3x10^5$ times higher than full sunlight) the short interval of exposure was not enough to allow the detection of F_m and it was more likely that FCM provided values that were between F_0 and F_m.

Finally, our data also showed that F_v/F_m correlated better with the FL of intact chloroplasts than with Pop A+B or damaged chloroplasts (Pop A). These correlations are especially meaningful considering that, despite the data obtained through PAM fluorometry measured on leaves containing chloroplasts in various physiological and structural states, they correlate better with the FL values provided by the intact chloroplasts population than with the FL of Pop A + B (all the chloroplasts).

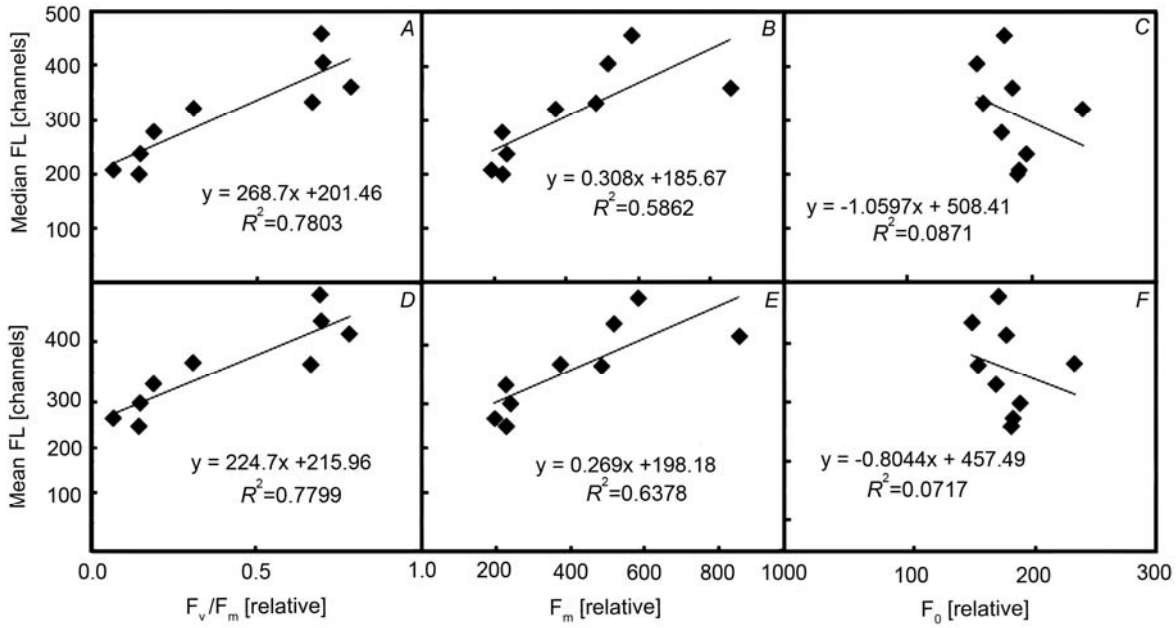

Figure 6: Correlation graphics between FL and F_v/F_m, F_m or F_0, for Pop. B. The graphics are relative to the median (*A*-F_v/F_m; *B*-F_m and *C*-F_0) and mean (*D*- F_v/F_m; *E*-F_m and *F*-F_0) FL value. Inserted in each graph is given the equation for the linear regression fitted to the data. The data obtained by PAM fluorometry was measured directly from intact leaves while FCM's data was collected from isolated chloroplasts.

The quantification of Chl *a* and *b* did not reveal significant effects caused by Paraquat and together with the results obtained with FCM and PAM we can state that the reduction in fluorescence parameters is due to the loss of chloroplasts integrity rather than by a decrease in Chl content. Reports like Franqueira *et al*., (1999) and Ekmekci and Terzioglu, (2005) might explain why we did not find significant variation of pigment content as a result of Paraquat exposure. Despite Franqueira *et al.* (1999) having found some variation within the first 24 h after exposure; only at 96 h were the effects significant. In the work of Ekmekci and Terzioglu (2005), pigment quantification was only performed after 48 h of exposure (all the other parameters were analyzed within the first 24 h) and in new leaves rather than developed ones, which might be consistent with the fact that Paraquat-induced effects in pigment content are only truly significant after 24 h. However other authors (Mascher *et al.,* 2005, Iturbe-Ormaetxe *et al*., 1998) have detected a significant decrease in pigment content within the first 8 h after Paraquat exposure. Unlike in our work, however, those authors samples were exposed to a much higher light intensity than the one used by us and Paraquat's effects require and are intensified by high light intensities (Varadi *et al.,* 2000). Considering the variations in volume detected by FCM (indicative of chloroplast burst) it is possible that in our work, chloroplast destruction occurred prior to degradation of chlorophyll.

The data presented here has demonstrated that the most sensitive endpoint to Paraquat exposure was the chloroplast's structure (both FS and SS) which showed significant differences at the first point of analysis (3 h); variation in fluorescence emission (either FCM or PAM) was only truly meaningful after 12 h of exposure. These results indicate that Paraquat causes a disruption of the chloroplast's integrity which provokes a gradual loss of function of the electron transport chain.

We also demonstrate that FL correlated significantly with F_v/F_m and better than with F_m or F_0; this information is an important contribution that helps to clarify contradictory reports claiming that FL had characteristics of F_0 or F_m.

In conclusion, FCM proved to be an effective and robust tool for assessing chloroplast functionality and it is expected that this investigation encourages researchers to explore the potentialities of this technique and provide new insights in photosynthesis.

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CHAPTER III-2

LEAD-INDUCED PHOTOSYNTHETIC IMPAIRMENT: BIOCHEMICAL PROCESSES ARE MORE SENSITIVE THAN THE PHOTOCHEMICAL APPARATUS

Chapter submitted as an original article to an SCI journal

Rodriguez E, Santos C, Azevedo R, Moutinho-Pereira J, Correia C and Dias MC.(2011). Lead-induced photosynthetic impairment: biochemical processes are more sensitive than the photochemical apparatus. Ecotoxicology and Environmental Safety (submitted).

Abstract

In order to evaluate Lead (Pb) induced toxicity on the photosynthetic apparatus, *Pisum sativum* L. plants were exposed to different Pb concentrations (from 20 to 2000 mg l^{-1}) during 28 days. The effects of Pb on the photosynthetic performance were analyzed by using the following endpoints, gas exchange parameters, chlorophyll *a* fluorescence, leaf pigments, RuBisCO activity and soluble sugars and starch content; also flow cytometry (FCM) was used to assess the morphological status and fluorescence emission status of chloroplasts.

The photosynthetic rate (*A*) and RuBisCO activity decreased strongly in Pb exposed plants while C_i/C_a increased as compared to control plants. The chlorophyll fluorescence parameters were not affected by Pb exposure, while the leaf pigment contents increased in plants exposed to 2000 mg l^{-1}. Soluble sugars showed a heterogeneous response to Pb exposure while starch content increased in plants exposed to 1000 and 2000 mg l^{-1}. The morphology of chloroplasts was affected by Pb exposure; the general trend shows a decrease in volume.

The results suggest that the reduction of the photosynthetic rate in Pb exposed plants were mainly due to biochemical process *e.g.* decreased RuBisCO activity as the photochemical apparatus seems to be resistant to Pb. We also prove the usefulness of FCM to provide Pb phytotoxicity endpoints, namely the ones related to morphological status of the chloroplasts.

Keywords: chloroplast morphology; lead toxicity; *Pisum sativum*; photosynthesis; RuBisCO; soluble sugars.

Introduction

Metal contamination is common in agricultural soils and water supplies mainly due to anthropogenic activities like industries, mining and fuel burnings. By this mean, metals have become a major environmental problem in the last decades.

The potential mobilization of metals in soil depends primarily on metal content, its solubility in water, soil pH, redox potential and other soil characteristics (Bertrand and Poirier, 2005). Lead (Pb), is one of the most widely distributed metal contaminants, being released from innumerous anthropogenic sources, being a major concern for human health and a considerable source of distress to the environment. Once in contact with plants, Pb is transported by CPx-type ATPases, a subgroup of P-type ATPases, that pump essential and non-essential metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} across the plasma membrane (Kučera *et al.*, 2008). It is known that Pb causes nutrient imbalance (*e.g.* Ca, Fe, Mg, Mn, P and Zn) by blocking the entry or binding the ions to ion-carriers making them unavailable for uptake and transport from roots to leaves (Xiong, 1997). This in turn may affect several physiological and biochemical processes, among which photosynthesis is one of the most important.

The deleterious effects of this metal in several physiological parameters have been addressed in some species: John *et al.*, (2009) found in *Brassica juncea* exposed to this metal, growth impairment and decrease in pigments content; Kosobrukhov *et al.*, (2004) working with *Platango major* showed that Pb can affect stomatal conductance (g_s) and pigment contents; Bibi and Hussain (2005) demonstrated that the photosynthetic rate (A), transpiration rate (E) and (g_s) of *Vigna mungo* plants were significantly affected when exposed to Pb. Parys *et al.*, (1998), found that Pb significantly affected the transpiration intensity, osmotic pressure of cell sap, water potential of xylem and relative water content of pea protoplasts after 24 and 48h of exposure. Unfortunately, even with these data, we still lack information about Pb phytotoxicity, which could enable a better understanding of the effects and mechanism of action of this metal and furthermore, help in the development of a more consistent model of Pb phytotoxicity at the photosynthetic level.

To fulfil that end, in the present investigation, pea plants were grown on soil and exposed to different Pb-containing solutions, with the concentrations ranging from 20 mg l^{-1} (maximum admitted concentration in water for agricultural purposes) to 2000 mg l^{-1}. After 28 days, several photosynthetic biomarkers were applied in order to comprehensively characterize the toxicity of Pb: quantification of soluble sugars and starch, leaf pigments, gas exchange parameters, chlorophyll a fluorescence and RuBisCO activity. Moreover, and for the first time, flow cytometry (FCM) was used to evaluate the effect of metals in the morphology and fluorescence intensity (FL) of chloroplast extracted from plants exposed to Pb.

Material and Methods

Plant culture conditions and exposure to lead

Pea seeds (*Pisum sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sawed in pots containing a peat:perlite mixture (4:1). Plants were grown during 28 days at 24ºC ± 1ºC, under light intensity of 200 µmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark).

Pb treatment consisted in watering plants (at least 25 per condition) during the aforementioned period of 28 days, twice per week, with 100 ml of a 1:10 Hoagland's solution containing the following concentrations of $PbCl_2$: 0; 20; 200; 1000 and 2000 mg l^{-1}. Afterwards plants were collected, rinsed thoroughly to remove substrate adhered to roots and tissue sampled for analysis. At least two independent experiments were performed for each parameter.

Lead and nutrient accumulation

Pb accumulation levels (roots and shoots), as well as those of some photosynthetic important nutrient (K, Mg, Mn, P, Zn, Fe) (n=3) were verified by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France). Roots were washed for 10 min in 0.5mM $CaSO_4$ to remove (by cation exchange) Pb^{2+} adsorbed to the root surface. Tissues were dried to constant weight at 60ºC and treated according to Santos *et al.*, (2002) for posterior analysis. The real Pb concentration in the growing media with nominal concentrations of, respectively, 0; 20; 200; 1000 and 2000 mg l^{-1} Pb, was also measured by ICP-AES.

Pigments quantification

Leaf disks (0.5 cm^2) were ground in a mortal at 0ºC with 2 ml cold acetone/Tris 50 mM pH 7.8 buffer solution (80:20, v:v) and centrifuged at 2,800 × *g* during 5 min. The supernatant was diluted to a final volume of 3 ml with additional acetone/Tris buffer. The absorbance at 470, 537, 647 and 663 were determined with a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S, Madison MI, USA). The contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids were calculated using the formulae of Sims and Gamon (2002). According to Lichtenthaler (1987), the molecular weights used to convert gram units to mole units were as follows: Chl *a* = 893.5 g mol^{-1}, Chl *b* = 907.5 g mol^{-1} and carotenoids = 550 g mol^{-1}.

Gas exchange and chlorophyll a fluorescence

Chlorophyll *a* fluorescence parameters were measured in the adaxial side of the leaf with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). The

maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v/F_m = (F_m-F_0)/F_m$, by measuring the fluorescence signal from a dark-adapted leaf when all reaction centers are open (using a low intensity pulsed measuring light source -F_0) and during a pulse saturating light (0.7 s pulse of 15,000 µmol photons m^{-2} s^{-1} of white light) when all reactions centers are closed (F_m). Leaves were dark-adapted for 30 min using dark-adapting leaf-clips for these measurements. Following F_v/F_m estimation, after a 20 s exposure to actinic light (1,500 µmol m^{-2} s^{-1}), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (15,000 µmol m^{-2} s^{-1}) for 0.7 s to establish F'_m. From these measurements, quantum effective efficiency of PSII was calculated as $\Phi_{PSII} = \Delta F/F'_m = (F'_m-F_s)/F'_m)$. Leaf gas exchange measurements were performed using a portable IRGA (LC*pro*+, ADC, Hoddesdon, United Kingdom), operating in the open mode under growth chamber conditions. Net CO_2 assimilation rate (*A*), stomatal conductance (g_s), transpiration rate (*E*) and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) were estimated from gas exchange measurements using the equations developed by Caemmerer and Farquhar (1981). The *A*/*E* ratio was used as instantaneous water use efficiency (WUE).

Gas-exchange and chlorophyll fluorescence measurements were always performed between 13:00 and 14:00 h (8 h after the beginning of the light period).

RuBisCO activity

Leaf discs of 0.5 cm^2 size were homogenized at 0 °C with 1 ml extraction buffer according to Dias and Brüggemann (2010). The extraction buffer consisted of 50 mM Tris/HCl, pH 7.9, 8 mM MgCl2, 5 mM Na-pyruvate, 1 mM EDTA, 2 mM K2HPO4, 20 mM dithiothreitol and 0.3 % (m/v) bovine serum albumin. The homogenate was centrifuged in at 9,000 × *g*. Immediately after extraction, ribulose 1,6 bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) activity was assayed as described by Lilley and Walker (1974). This assay followed NADPH oxidation measured spectrophotometrically at 340 nm. Total activity was achieved after incubation in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min.

Soluble sugars and starch

Soluble sugars were extracted from frozen leaf discs (0.5 cm^2) with 2 ml 80% (v/v) of ethanol at 80ºC over 20 min as described by Correia *et al.*, (2005). Glucose, fructose and sucrose were quantified using a spectrometric enzyme-coupled assay described by Jones *et al.*, (1977); starch was quantified according to Stitt *et al.*, (1978). All of the analysis were performed in a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S, Madison MI, USA).

Chloroplast morphology and fluorescence emission by FCM

For FCM analysis, chloroplast extraction and analysis was performed according to Rodriguez *et al.*, (2011). In brief, leaves were harvested as needed and grinded with a mortar and pistil, in HS buffer (HEPES-sorbitol buffer). The suspension was filtered through 50 µm nylon meshes and centrifuged for 2 min at 4,000 × *g*. Supernatant was collected and layered on 2 ml of a 35% Percoll® solution and centrifuged for 8 minutes at 1,400 × *g*. The pellet was collected, re-suspended and centrifuged twice in HS buffer at 4,000 × g for 2 min and then analyzed in a Coulter Epics XL flow cytometer (Beckman Coulter Inc, Hialeah Miami, FL). The forward angle light scattered (FS- corresponding to the volume) of each particle as well as the relative fluorescence intensity (FL) emitted by each chloroplast were collected and analyzed, being that at least three plants were sampled by condition (and run in triplicates). From the conjunction of the FL histogram and the FS vs SS (side angle light scatter) cytogram of control chloroplast, 2 regions were defined to discriminate between intact chloroplast (high FL) and broken/damaged chloroplast (low FL). After this, the voltage of the cytometer and the regions characterizing these populations were kept constant for the remaining of the assay.

Data analyses and statistics

Gas exchange and chlorophyll fluorescence data are averages of individual determinations of 5-7 plants. Pigments, soluble sugars, starch and RuBisCO activity data are averages of individual measurements in 4-5 plants. The results were analyzed with a one-way analysis of variance (ANOVA), using the Sigma Stat program for Windows, version 3.5. Pairwise comparisons between means were evaluated by post hoc Holm-Sidak multiple comparison test.

Results

Survival rate and growth

After 28 days of culture, control and Pb treated plants showed similar survival rates (100%). Morphologically, both plants looked similar, with well developed leaves and without necrotic or chlorotic symptoms.

The variation in fresh and dry weights is presented in Table 1. For shoots, the only concentration which significantly ($P \leq 0.05$) affected both parameters was 2000 mg l^{-1}; at this concentration, the fresh weight decreased 29 % and the dry weight 43 %, in respect to control. Roots were more sensitive to Pb toxicity than shoots; while 2000 mg l^{-1} caused the most substantial decrease (68 %), plants exposed to 1000 mg l^{-1} also displayed a significant decrease in dry weight (61 %), in respect to control plants (Table 1). Relatively to the fresh weight, the highest dosage was the only Pb treatment capable of inducing significant decrease in roots (42 %), in respect to control values.

Table 1 Fresh (FW) and Dry (DW) weight variation in shoots (s) and roots (r) from control and Pb-exposed plants. Values given are the mean % variation in respect to control's weight ± SD (%) of the values. Values followed by different letters indicate significant differences between treatments ($P \leq 0.05$)

Pb (mg l^{-1})	FW s (%)	DW s (%)	FW r (%)	DW r (%)
0	0 ± 6.1^{a}	0 ± 0.50^{a}	0 ± 1.53^{a}	0 ± 0.18^{a}
20	5 ± 1.5^{a}	1 ± 0.21^{a}	7 ± 3.58^{a}	19 ± 0.37^{ab}
200	8 ± 7.3^{a}	20 ± 0.39^{ab}	30 ± 0.99^{ab}	32 ± 0.28^{ab}
1000	17 ± 1.4^{ab}	23 ± 0.27^{ab}	23 ± 2.11^{ab}	61 ± 0.17^{b}
2000	29 ± 3.9^{b}	43 ± 0.14^{b}	42 ± 1.03^{b}	68 ± 0.09^{c}

Pb and nutrient quantification

The Pb concentration in the control (0 mg l^{-1}) medium was below the ICP-AES detection limit. In other growing media with Pb nominal concentrations of 20; 200; 1000 and 2000 mg l^{-1}, the measured values were, respectively, of 20.07 $\pm$ 0.004 mg l^{-1}; 200.09 $\pm$ 0.020 mg l^{-1}; 1000.13 $\pm$ 0.055 mg l^{-1}; 2000.26 $\pm$ 0.95 mg l^{-1} of total Pb.

Pb accumulation data indicated that most of the metal was accumulated in the roots and only a small fraction was translocated to the shoots. In fact, the ratio between Pb accumulated in roots and leaves of the same plant (condition) starts with 1.5 times in control plants and then rose to 11 fold at 20 mg l^{-1} peaking around 35 fold at the highest concentrations (1000 and 2000 mg l^{-1}). These data also demonstrate that the amount of Pb accumulated in both tissues increased with the increasing concentration of this metal in the growth medium (Table 2).

Table 2 Lead content (µg g^{-1} DM) in shoots and roots of control and Pb-treated plants. Values are given as means ± SD. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Pb (mg l^{-1})	Pb in shoots (µg g^{-1} DM)	Pb in roots (µg g^{-1} DM)
0	9.0 ± 1.3^{a}	13.6 ± 10.1^{a}
20	58.8 ± 10.3^{b}	613.1 ± 60.3^{b}
200	65.4 ± 7.9^{b}	1139.6 ± 238.9^{b}
1000	65.9 ± 8.6^{b}	2431.5 ± 422.1^{c}
2000	109.9 ± 7.7^{c}	3477.2 ± 255.5^{d}

Tissues from plants exposed to any of the solutions containing Pb presented significantly higher levels of this metal than control plants ($P \leq 0.05$), being that the highest levels of Pb accumulated were observed in tissues from plants exposed to 2000 mg l^{-1}.

Exposure to Pb affected the accumulation of nutrients in roots and shoots, which can be seen in Table 3. In roots, control's K, P and Mn levels were significantly higher than those of Pb treated plants ($P \leq 0.05$); on the other hand, the amount of Fe in controls was significantly lower ($P \leq 0.05$) that those of the other conditions .

For shoots, some of the trends observed in roots were maintained (decrease in P, Mn and increase in Fe, when exposed to Pb) but new imbalances were also observed. The level of K, unlike what was observed in roots, increased ($P \leq 0.05$) in shoots of plants exposed to the two highest concentrations of Pb. Also, the levels of Mg and Zn increased substantially in plants exposed to Pb.

Table 3 Nutrient accumulation (µg g^{-1} DM or mg g^{-1} DM) in tissues from control and Pb-treated plants. Values are presented as means ± SD. Different letters indicate significant differences between treatments ($P \leq 0.05$).

	Pb(mg l^{-1})	K(mg g^{-1})	Mg(mg g^{-1})	Mn(µg g^{-1})	P(mg g^{-1})	Zn(µg g^{-1})	Fe(mg g^{-1})
Roots	0	40.4±8.5^{a}	2.66±0.1^{a}	265.87±39.1^{a}	11.64 ± 1.305^{a}	114.89±16.7^{a}	0.23 ± 0.050^{a}
	20	12.6±12.8^{b}	2.80±2.4^{a}	98.30±22.8^{b}	3.43 ± 1.529^{b}	203.90±95.0^{a}	1.60 ± 0.910^{b}
	200	14.7±14.5^{b}	4.97±4.1^{a}	92.33±13.4^{b}	2.61 ± 1.308^{b}	210.39±115.6^{a}	2.95 ± 0.236^{c}
	1000	12.9±4.5^{b}	7.57±6.0^{a}	87.53±8.3^{b}	3.013 ± 0.725^{b}	266.79±228.8^{a}	2.55 ± 1.076^{c}
	2000	22.1±9.9^{b}	4.41±2.8^{a}	55.03±12.3^{c}	4.09 ± 0.676^{b}	171.47±34.3^{a}	1.40 ± 0.113^{b}

	Pb(mg l^{-1})	K(mg g^{-1})	Mg(mg g^{-1})	Mn(µg g^{-1})	P(mg g^{-1})	Zn(µg g^{-1})	Fe(mg g^{-1})
Leaves	0	41.2±2.5^{a}	1.58±0.1^{a}	62.20±5.3^{a}	8.65 ± 0.324^{a}	37.98±3.4^{a}	0.15 ± 0.037^{a}
	20	40.1±2.9^{a}	3.08±0.9^{b}	42.80±4.1^{b}	6.33 ± 1.969^{b}	61.06±8.7^{b}	0.66 ± 0.317^{b}
	200	38.9±2.2^{a}	3.20±0.7^{b}	43.79±7.1^{c}	5.02 ± 0.589^{b}	68.17±6.0^{b}	0.60 ± 0.208^{b}
	1000	53.1±3.5^{b}	3.32±1.3^{b}	54.69±2.2^{d}	5.94 ± 0.853^{b}	74.58±21.7^{b}	1.30 ± 0.221^{c}
	2000	59.0±8.4^{b}	3.12±0.8^{b}	49.57±2.4^{c}	6.54 ± 2.461^{b}	73.75±15.2^{b}	0.55 ± 0.082^{b}

Chlorophyll and carotenoids

Figure 1 presents the quantification of Chl *a*, Chl *b* and carotenoids, revealing that these pigments presented a highly similar pattern in response to Pb treatment. At 2000 mg l^{-1}, a high and significant increase (the only significant change observed) in pigment content was observed ($P \leq 0.05$), representing augments of 42, 62 and 57 % for Chl *a*, Chl *b* and carotenoids, respectively (when compared to control). Relatively to the Chl *a*/Chl *b* ratio, this parameter suffered little variation ($P > 0.05$) among the conditions between control and 1000 mg l^{-1} but was 35 % lower in plants exposed to 2000 mg l^{-1} ($P \leq 0.05$).

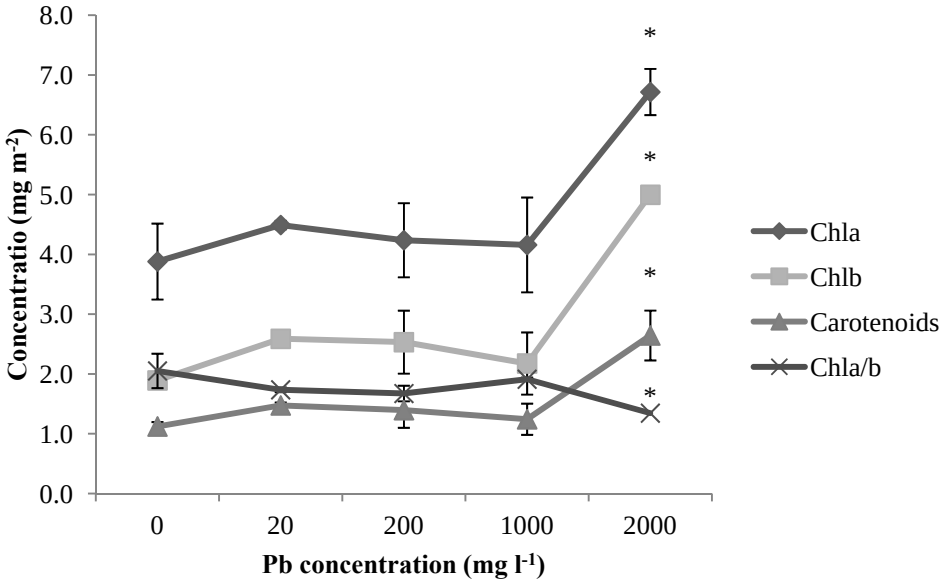

Figure 1 Pigment content variation of leaves from plants exposed to given concentration of Pb. Pigments are Chlorophyll a (Chl a) Chlorophyll b (Chl b) and Carotenoids. The Chl a/Chl b ratio is also given (Chl a/b). The data is presented as the mean and standard deviation of the mean. Values followed by (*) are significantly different from control ($P \leq 0.05$)

Chlorophyll a fluorescence

The F_v/F_m in control plants was 0.842 ± 0.006 (mean ± sd). Exposure to Pb caused little variation ($P > 0.05$) in this parameter, values ranging from 0.815 ± 0.016 (20 mg l^{-1}) to 0.829 ± 0.022 (1000 mg l^{-1}) (Table 4). The Φ_{PSII} was also determined, but Pb exposure did not affect this endpoint significantly, at any of the concentrations assayed ($P > 0.05$). Control plants presented a mean Φ_{PSII} of 0.795 ± 0.061 while the lowest and maximum values verified in plants exposed to Pb were verified at 20 mg l^{-1} (0.729 ± 0.058) and 2000 mg l^{-1} (0.802 ± 0.026) respectively (Table 4).

Table 4 Maximum photochemical efficiency of PSII (Fv/Fm), and quantum effective efficiency of PSII (ΦPSII) from leaves of pea plants exposed to given concentration of Pb. Data is presented as mean and standard deviation of the mean. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Pb(mg l^{-1})	F_V/F_M	SD	ΦPSII	SD
0	0.84[a]	0.006	0.80[a]	0.060
20	0.81[a]	0.015	0.73[a]	0.058
200	0.83[a]	0.074	0.77[a]	0.118
1000	0.83[a]	0.022	0.74[a]	0.053
2000	0.82[a]	0.049	0.80[a]	0.026

Gas-exchange parameters

Pb exposure significantly affected most of the markers used to evaluate the gas-exchange endpoint. The *A* suffered a steady decrease from control to 200 mg l^{-1} (Figure 2), and beyond that dosage, values decrease in a more subtle manner. The mean values for any given treatment were significantly lower than that of the controls ($P \leq 0.05$). The lowest value was observed at 1000 mg l^{-1}, being 4.5 fold lower than control's.

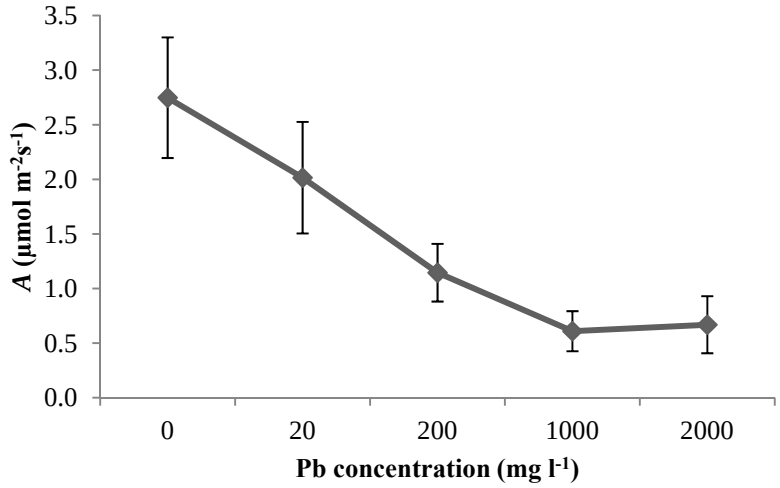

Figure 2 Variation of the photosynthetic rate (*A*) of pea plants exposed to Pb. The values are given as the mean ± standard deviation of the mean. All values are significantly different from control ($P \leq 0.05$).

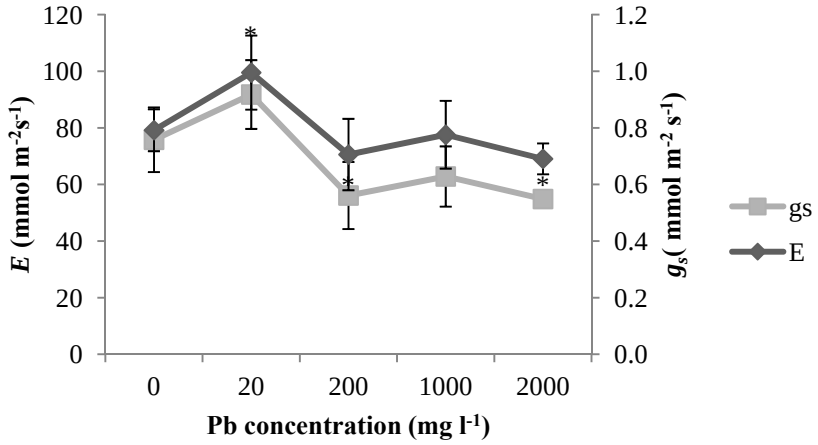

Figure 3 Transpiration rate (*E*, on the left axis) and stomatal conductance (g_s on the right axis) of pea plants exposed to given concentration of Pb. The values are the mean ± standard deviation of the mean. Values followed by (*) are significantly different from control ($P \leq 0.05$)

The *E* was significantly higher in plants exposed to 20 mg l^{-1}, when compared to the remaining conditions ($P \leq 0.05$), which presented *E* of the same order as control (Figure 3). The g_s decreased significantly in plants exposed to 200 and 2000 mg l^{-1} of Pb when compared to control plants ($P \leq 0.05$), presenting a very similar pattern to that observed for *E* (Figure 3).

As for the WUE, this endpoint was one of the most affected by Pb exposure; any Pb concentration caused a significant decrease in this parameter ($P \leq 0.05$). Furthermore, at the two highest concentrations, the WUE was significantly lower than for 20 and 200 mg l^{-1} ($P \leq 0.05$) (Figure 4). The maximum decrease in this parameter was observed from control to 1000 mg l^{-1}, with a decrease of nearly 80 %.

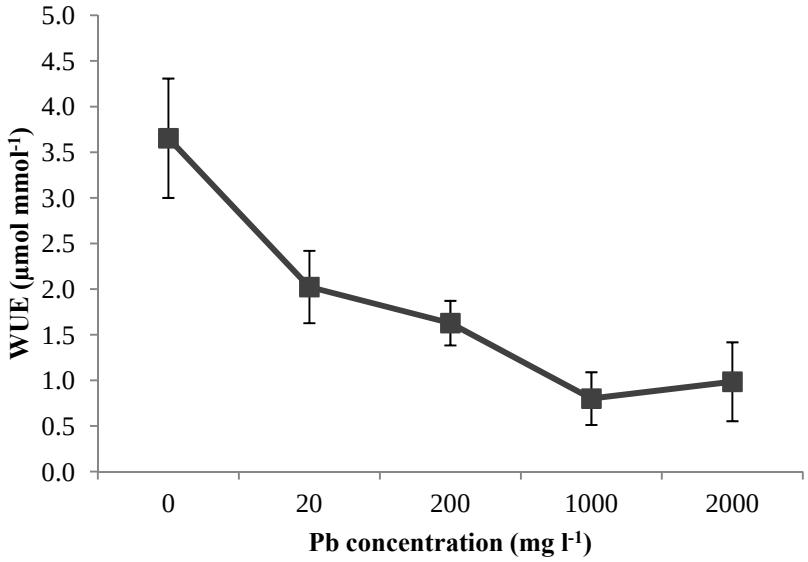

Figure 4 Instantaneous water use efficiency (*WUE*) changes in control and plants exposed to Pb. The values are given as the mean and standard deviation of the mean; all values are significantly lower than the control's ($P \leq 0.05$)

Relatively to the C_i/C_a, control plants presented the lowest value (0.866 ± 0.051) observed and exposure to Pb caused a steady increase in this parameter. However, the only values that were significantly higher than control's were those from plants exposed to 1000 mg l^{-1} and 2000 mg l^{-1} ($P \leq 0.05$), with an increase of nearly 10 % (Figure 5).

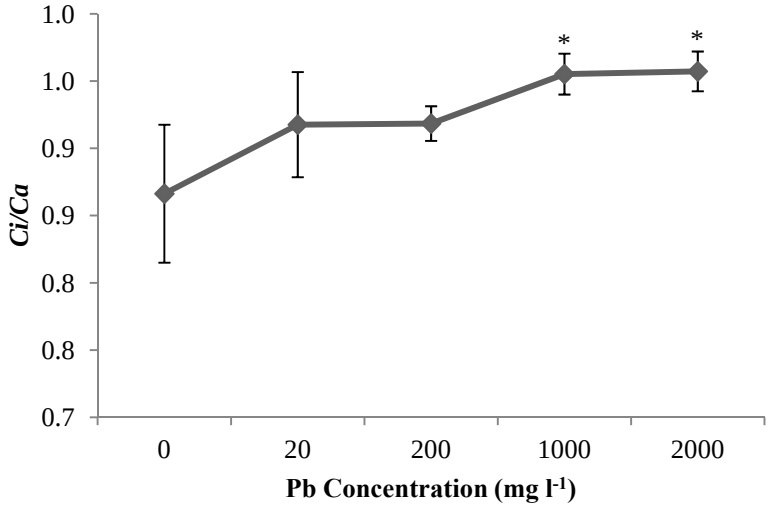

Figure 5 Intercellular to atmospheric CO_2 ratio of plants exposed to given concentration of Pb. The values are given as the mean and standard deviation of the mean. Values followed by (*) are significantly different from control ($P \leq 0.05$)

Calvin cycle enzymes

The evaluation of Pb exposure effect on RuBisCO showed that this enzyme's activity decreased even at the lowest dose used, 20 mg l^{-1}, being 2.15 fold lower at this concentration. The activity of RuBisCO increased slightly from 20 to 1000 mg l^{-1}, but it was still significantly lower than the control's one (Figure 6). Plants exposed to 2000 mg l^{-1} had the lowest RuBisCO activity (decrease of 2.6 fold in respect to control plants), being significantly lower than all conditions ($P \leq 0.05$) except of 20 mg l^{-1} ($P > 0.05$).

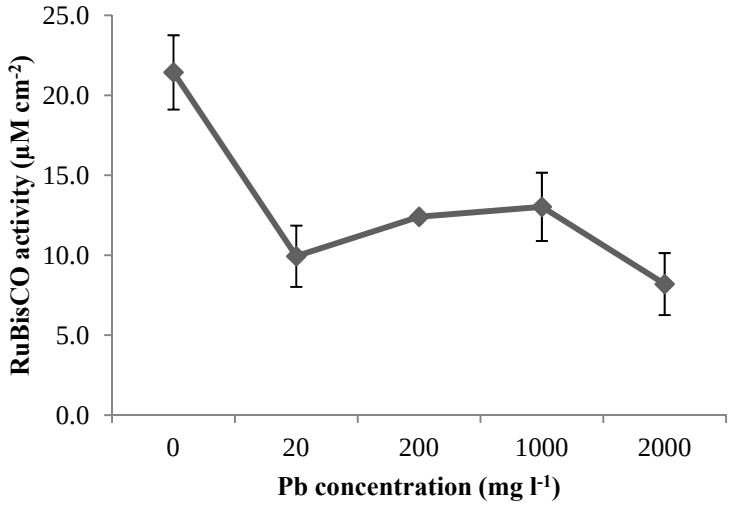

Figure 6 Maximum RuBisCO activity of leaves from control and Pb exposed plants. The values are given as the mean and standard deviation of the mean. Values are significantly lower than the control's ($P \leq 0.05$)

Soluble sugars and starch

The effect of Pb in the quantity of soluble sugars and starch produced heterogeneous results. The amount of glucose rose to its maximum at 200 mg l^{-1} (1.8 fold increase to control $P \leq 0.05$), followed by a slight decrease in plants exposed to 1000 mg l^{-1} (1.5 times higher than control $P \leq 0.05$) and 2000 mg l^{-1} (not significantly different from control).

Like for glucose, the maximum concentration of fructose was verified in plants exposed to 200 mg l^{-1} of Pb (2.7 fold increase $P \leq 0.05$), followed by a harsh decrease at 1000 mg l^{-1} (almost 50 % decrease from 200 mg l^{-1}). Exposure to 2000 mg l^{-1} raised fructose concentration in 24 % (in respect to 1000 mg l^{-1}) to a net value of 107 ± 19.68 µM cm^{-2} (Figure 7), which was significantly higher than control ($P \leq 0.05$)

Sucrose concentration in control plants was 120.4 ± 22.40 µM cm^{-2} and Pb exposure caused a steady increase up to 181.9 ± 19.36 µM cm^{-2} in plants exposed to 1000 mg l^{-1} ($P \leq 0.05$), an increase of 33 % in respect to control. Plants exposed to 2000 mg l^{-1} had a mean concentration of sucrose 59.4 ± 18.24 µM cm^{-2}, representing a 3 fold decrease ($P \leq 0.05$) in respect to plants exposed to 1000 mg l^{-1} and 1.5 fold lower than what was verified for control plants ($P \leq 0.05$).

As for starch, the concentration of this sugar remained constant from control to plants exposed 200 mg l^{-1} of Pb ($P > 0.05$). At 1000 and 2000 mg l^{-1} a significant increase of 40 % and 43 % (respectively) in the concentration of starch was observed ($P \leq 0.05$), when compared to control plants.

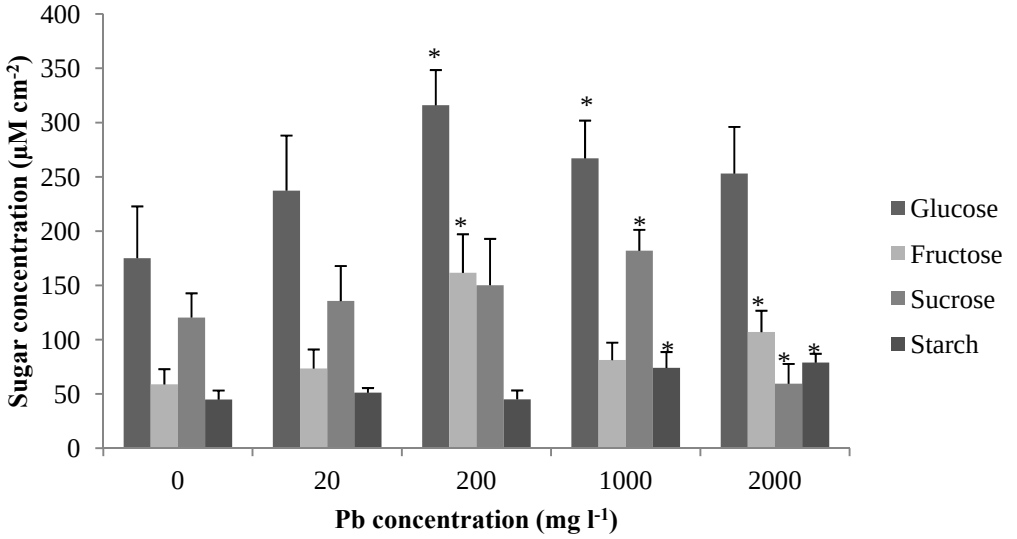

Figure 7 Lead effect on the soluble sugars and starch content of leaves from control and exposed plants. Soluble sugars measured are glucose, fructose and sucrose. The values are presented as

the mean and standard deviation of the mean. Values followed by (*) are significantly different from control ($P \leq 0.05$)

FCM evaluation of chloroplast morphology and fluorescence

FCM was used to evaluate changes in FS of chloroplast extracted from control and plants exposed to Pb. The volume of the chloroplast decreased with Pb exposure and significant differences were observed for plants exposed to 1000 mg l^{-1} (decrease of 21 %) and 2000 mg l^{-1} (16 %), when compared to control plants (Table 5).

As for the relative fluorescence (FL) of chloroplast, only plants exposed to 1000 mg l^{-1} showed significant differences ($P \leq 0.05$), with the mean value being 20 % lower than the control's.

Table 5 Changes in volume (FS) and fluorescence emission (FL) of chloroplast extracted from pea plants exposed to given concentration of Pb. Values are the mean and standard deviation of the mean. Values followed by the different letters are statistically different ($P \leq 0.05$)

Pb (mg l^{-1})	FS	SD	FL	SD
0	55.5^{a}	4.02	257.8^{a}	4.61
20	50.4^{ab}	3.68	245.8^{a}	10.66
200	48.8^{ab}	1.60	258.7^{a}	6.20
1000	43.7^{b}	3.22	206.5^{b}	4.21
2000	46.5^{b}	0.64	253.7^{a}	9.25

Discussion

Pb accumulation and nutrient imbalance

Pb assimilation and nutrient imbalances are among the first steps in this metal's mechanism of phytotoxicity. Despite that the mechanism by which Pb enters in the roots is still unknown (Peralta-Videa *et al.*, 2009), some evidence (Kučera *et al.*, 2008) suggests that it is transported by CPx-type ATPases. It is also thought that Pb competes with other elements for root uptake, namely Ca^{2+}. Even though we did not found evidence of Ca^{2+} uptake impairment in roots, (data not shown) we verified that K, P, and Mn levels significantly decreased in roots exposed to Pb. These results are consistent with the decrease in K and Mn (among others) observed by Breckle and Kahle (1992), in beech exposed to this metal. However, we also observed a significant increase in Fe accumulation in plants exposed to Pb whilst those authors detected the opposite. It has been proven in human cells (Yanagiya *et al.*, 2000) that Cd and Mn compete for cellular uptake and due to the fact that CPx-type ATPases are thought to transport both metals (and others), it is possible that the decrease in Mn was caused by direct competition with Pb. As for the decrease in

K, this might be explained by a perturbation of the homeostasis of the cells, rather than by direct competition of Pb.

Gas exchange parameters and RuBisCO activity

A detrimental effect of Pb on the photosynthetic parameters (*A*, *E*, g_s and C_i/C_a) was reported by several authors in different species (Krupa and Baszynski, 1995, Prasad, 1996, Bibi and Hussain, 2005, Kaznina *et al.*, 2005). In the present work, Pb exposure led to a pronounced reduction of the *A,* mainly due to non-stomatal limitations, although increased stomatal resistance was also observed. In fact, an increase of C_i/C_a values in pea plants exposed to Pb (more markedly under 1000 and 2000 mg l^{-1} of Pb) suggests that the intercellular CO_2 concentration available in the mesophyll cells might not be the major limiting factor for photosynthesis. Limitation of photosynthesis by reduced activity of the Calvin cycle enzymes, *e.g.* RuBisCO activity was reported for several plant species exposed to Pb (Vojtěchová and Leblová, 1991, Moustakas *et al.*, 1994, Parys *et al.*, 1998). The strong reduction of the maximal RuBisCO activity (69, 42, 39 and 63% less than controls, respectively for 20, 200, 1000 and 2000 mg l^{-1} of Pb) observed in *P. sativum* plants may explain the decrease of the *A*. Lee and Roh (2003) found that exposure of *Canavalia ensiformis* to Cd induced significant decrease in RuBisCO activity, which was associated to the amount of RuBisCO protein. Due to the similarity between the mechanism by which Pb and Cd induce phytotoxicity at this level, the results observed by those authors might be a valid hypothesis to justify the decrease in RuBisCO activity observed in our Pb-treated plants.

Another photosynthetic endpoint of Pb toxicity in *P. sativum* was the decrease in WUE. This parameter was strongly affected by Pb exposure being that the decrease observed for this endpoints was correlated with the aforementioned reduction of the *A* (PC = 0.966 and P = 0.00735).

Pigment content

The pigment content of the leaves was not significantly affected by most Pb concentrations. A significant increase was observed at the highest dose with Chl *a* increasing 42 %, Chl *b* 62 % and carotenoids 58 %. The high pigments content suggests that Pb exposure did not impair the core complexes of PSII (Choudhury and Behera, 2001). This is also congruent with the low variation of chlorophyll fluorescence values observed among control and treated plants. Carotenoids play a key role in protecting chlorophyll pigments under stress conditions (Choudhury and Behera, 2001). The high levels of this pigment in Pb exposed plants, in particular those at 2000 mg l^{-1}, may reflect a protection of the photosynthetic machinery from photo-oxidative damage. These findings are in agreement with data presented by Zeng *et al.*, (2006), where rice plants exposed to Pb displayed significant increase in pigment content when compared to control. They hypothesized that up to certain doses, Pb could cause an improvement of soil's nutrition conditions.

John *et al.*, (2009) reported that *Brassica* plants exposed to 150 μM Pb (6.32 mg of Pb accumulated per g of DM in leaves) presented higher pigment levels than control, while higher doses progressively led to a decrease in pigment contents. Our plants accumulated 0.1 mg of Pb per g DM, so it is possible that with this level of Pb accumulation, the quantity of pigments increases rather than decrease. Parys *et al.*, (1998) did not found changes in pigment content in excised leaves of pea exposed to Pb, although this could be justified with the short period of exposure (24 and 48h) used in the experiment, which might not be sufficient to induce significant changes in pigment content. Cenkci *et al.*, (2010) on the other hand, found a negative correlation between Pb exposure and pigment content in turnip leaves but unlike in our pea plants, they observed a decrease of the shoot Pb/ root Pb accumulation ratio, from 27 fold to 1.77 fold (lowest to highest dosage). This higher accumulation/translocation of Pb in turnip plants when compared to pea could be the reason for a decrease in pigments on the former but not in the latter.

Despite of the increase in pigment content at 2000 mg l^{-1} Pb, we found a significant decrease in Chl *a*/Chl *b* ratio of 35 % in respect to control plants. Li *et al.*, (2011) suggested that an increase of the Chl *a*/Chl *b* ratio could be understood as an adaptation to endure stress conditions; in our case, the decrease in this ratio might be indicative of the opposite: the onset of deleterious effect to the photosynthetic apparatus.

Chlorophyll a fluorescence

Relatively to the fluorescence endpoint, we did not found significant alterations caused by Pb exposure, in most of the biomarkers assessed. This suggests that the photochemical apparatus of *P. sativum* is very resistant to Pb, at least in the range of concentrations tested. These results are in agreement with the reports of KrishnaRaj *et al.*, (2000) and Ruley *et al.*, (2006) in *Pelargonium* sp. and *Sesbania drummondii* respectively, where F_v/F_m values were not significantly affected by Pb accumulation. Also, in cucumber plants, Burzynski and Klobus, (2004) found that while the F_v/F_m was not affected by Pb toxicity, the Φ_{PSII} was significantly reduced in response to this metal. A possible justification to the fact that the photosynthetic apparatus of our plants was not significantly affected by Pb might be related with the findings of Aravind and Prasad (2004). Those authors demonstrated that Zn supplements alleviated the toxicity of Cd to the photosynthetic apparatus of *Ceratophyllum demersum*. In our case, shoots exposed to any of the Pb dosage tested accumulated significantly higher levels of Zn than control plants and it is therefore likely that this is part of the plant's strategy to protect its photosynthetic apparatus from Pb-induced damage.

Chloroplast structural and functional status

In an innovative study, Rodriguez *et al.*, (2011) demonstrated the suitability of FCM to measure variation in FL caused by Paraquat and successfully demonstrated a strong and positive correlation between FL and F_v/F_m. For the most part, in the present work both F_v/F_m and FL

presented similar results. We did however find a significant decrease in the FL of plants exposed to 1000 mg l^{-1}. The justification for this decrease is unclear, as the other biomarkers of fluorescence emission did not present significant differences. A justification to why this decrease was not observed at 2000 mg l^{-1} might be due to the observed increase in pigment content at that concentration, thus increasing the level of fluorescence emission.

The characterization of Pb effect on chloroplast morphology demonstrated that this metal caused a general alteration in the volume; the general trend of volume decrease which is mostly seen at higher concentrations, might be explained by an osmotic stress. The disarrangement of the granna caused by both Pb accumulation and insufficient amount of Mn (Clijsters and Assche, 1985) might also be a good explanation for our results even more so that this observation also fits with the impairment in Mn accumulation observed in our Pb treated plants.

Soluble sugars and starch

The last set of endpoints of Pb toxicity was the amount of soluble sugars and starch accumulated in leaves. The principal end products of photosynthesis are sugars. Under stress conditions, the increase of soluble sugar and others solutes, as proline, can be used to maintain leaf cell turgor and act as nutrient and metabolite signaling molecules (Rolland *et al.*, 2006). The high amount of soluble sugars (mainly glucose and sucrose) observed in pea plants exposed to Pb may contribute to the maintenance of cell turgor and to protect cell membranes against oxidative stress. The storage of starch in plants exposed to 1000 mg l^{-1} and 2000 mg l^{-1} might reflect an accumulation of this reserve carbohydrate in response to the stress condition.

Environmental perspective

Within the European Union, some countries have restrictive values for the maximum admitted level of metals in agricultural purposes waters. For Pb, this value is 20 mg l^{-1}, which was chosen as the lowest dosage tested in the present investigation. A sum of our results show that even at this dosage, some biomarkers were significantly affected (*i.e*. *A*, *WUE* and C_i/C_a), demonstrating that low dosages can still have deleterious effect to plant performance and that more restrictive limits should be considered. In contaminated sites, Pb levels can be several orders of magnitude higher; for instance at the Alta mine site at Montana (US), Pb levels almost reached 8000 ppm. The highest dosage we evaluated (equivalent to 2000 ppm) is well below those values and yet, it caused a dramatic decrease in many endpoints.

Conclusion

In conclusion, this study demonstrated that Pb exposure severely affect *P. sativum* photosynthetic status. The results suggest that the photosynthesis impairment of *P. sativum* plants

exposed to Pb was mainly due to biochemical processes (*e.g.* RuBisCO activity). The photochemical apparatus on the other hand seems to be very resistant to Pb, as demonstrated by the result provided by PAM fluorometry and FCM. We also proved the usefulness of FCM to provide Pb phytotoxicity endpoints, namely the ones related to morphological status of the chloroplasts.

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CHAPTER III-3

CHROMIUM (VI) INDUCES TOXICITY AT DIFFERENT PHOTOSYNTHETIC LEVELS IN PEA

Chapter submitted as an original article to an SCI journal

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Chromium (VI) induces toxicity at different photosynthetic levels in pea . Ecotoxicology and Environmental Safety (submitted).

Abstract

In order to comprehensively characterize the effects of Cr(VI) on the photosynthetic performance of *Pisum sativum* L., plants watered with Cr solutions (ranging from 20 to 2000 mg l^{-1}) were evaluated using the following classical endpoints: gas exchange parameters, chlorophyll *a* (Chl *a*) fluorescence, leaf pigments, RuBisCO activity, soluble sugars and starch content. Flow cytometry (FCM) was applied in an innovative approach to evaluate the morphological and fluorescence emission status of chloroplasts from plants exposed to Cr(VI). The biomarkers related to gas exchange, photosynthetic rate (*A*) and RuBisCO activity were severally affected by Cr exposure, in some cases even at the lowest dosage used. While all biomarkers used to measure Chl *a* fluorescence indicated a decrease in fluorescence at the maximum dosage, pigment contents significantly increased in response to Cr(VI). The morphology of chloroplasts suffered alteration due to Cr(VI) exposure, with a significant decrease in chloroplast volume being observed. Soluble sugars and starch showed an overall tendency to increase in Cr(VI) exposed plants, but sucrose and glucose decreased highly when exposed to 2000 mg l^{-1}. In conclusion, our results indicate that Cr(VI) affects photosynthesis at several levels but the most Cr(VI)-sensitive endpoints were chloroplast morphology and biochemical processes; only at higher dosages the photochemical efficiency is compromised.

Keywords: chloroplast morphology; chromium; photosynthesis; RuBisCO activity; soluble sugar

Introduction

Chromium (Cr) is one of the most abundant elements in earth crust (Subrahmanyam, 2008) and also due to its release in the environment by several industries (metallurgical, leather, and chemical) it has become an important metal pollutant (Pandey *et al*., 2009, Montes-Holguin *et al*., 2006). Shanker *et al*., (2005) stated that unlike other heavy metals (*e.g.* Cadmium- Monteiro *et al*., 2009), Cr had not received the same level of attention from plant scientist. Since then, the overall scenario regarding Cr phytotoxicity has changed very little; few reports have addressed the deleterious effects of this metal in plants and most of what is known comes from other organisms like animals and algae (*e.g.* Su *et al*., 2005, Oliveira *et al*., 2010, Rodriguez *et al*., 2011c).

Chromium phytotoxic potential depends on its speciation which is responsible for its mobilization, subsequent uptake and resultant toxicity (Shanker *et al*., 2005). Lopez-Luna *et al*., (2009) demonstrated, for 3 species of cereals, that Cr (VI) presented higher mobility and caused more toxic effects at lower levels than Cr (III) and tannery sludge (high Cr content). This is justified by the fact that Cr (VI) has higher solubility in water and enters cell membranes easily than other Cr valences (Rodriguez *et al*., 2007). Once inside the cell, Cr (VI) is known to generate reactive oxygen species (Dixit *et al*., 2002) and its intracellular reduction to Cr (III) enables a direct interaction with the DNA (Beyersmann and Hartwig, 2008) which might cause DNA degradation and cell cycle arrest (Rodriguez *et al*., 2011b), among others.

The little information available indicates that Cr can affect the plant's photosynthetic performance: in pea seedlings it was demonstrated that Cr exposure reduced the photosynthetic rate and the authors hypothesized that Cr could act as an acceptor of electrons during photophosphorylation, inhibiting the process (Bishnoi *et al*., 1993). Later, Vernay *et al*., (2007) verified that in *Lolium perenne*, Cr(VI) exposure affected the photochemistry of PSII and decreased CO_2 assimilation. More recently Lopez-Luna *et al*., (2009) showed that in sorghum, wheat and oat plants, significant translocation of Cr (VI) occurred to the aerial parts, supporting the possibility of interactions between this Cr valency and the photosynthetic machinery/process. In algae, Juarez *et al*., (2008) observed changes in chloroplast matrix and morphology, together with a complete pheophytinization of both chlorophyll *a* (Chl *a*) and *b* (Chl *b*) when exposed to Cr(VI).

Even with these evidences, there is still much to be done in order to fully understand the extent and effects of this pollutant in the photosynthetic process.

The aim of the present research was to analyze the effects of Cr(VI) in several aspects of photosynthesis, using pea as model crop species. To this end, plants were exposed to several Cr(VI) concentrations ranging from the maximum admitted level in some European Union countries for agricultural purpose waters (20 mg l^{-1} for total chromium) to values observed in waters from chrome tanning processes (2000 mg $l^{-1}$of Cr VI- Iyer and Mastorakis, 2006). The following biomarkers were used to assess Cr(VI)'s toxicity in photosynthesis: gas exchange parameters, photosynthesis-related nutrients unbalances, Chl *a* fluorescence, leaf pigments, RuBisCO activity, soluble sugars and starch contents. Also FCM was used as an emerging technique in

photosynthetic studies, in order to evaluate the morphological and fluorescence emission status of chloroplast extracted from plants exposed to Cr.

Material and Methods

Plant culture conditions and exposure to Chromium

Pea seeds (*Pisum sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sawed in pots containing a peat:perlite mixture (4:1). Plants were grown for 28 days at 24ºC ± 1ºC, under light intensity of 200 µmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark). Exposure to Cr(VI) was performed by watering plants (at least 25 per condition) twice per week, with 100 ml of a 1:10 Hoagland's solution containing the following concentrations of $K_2Cr_2O_7$: 0; 20; 200; 1000 and 2000 mg l^{-1}. The exposure lasted the aforementioned period of 28 days, after which analyses were performed.

Chromium and nutrient analysis

Contents of total Cr and of some nutrients essential to photosynthesis (Mg, Mn, Fe, S, P) were determined in dried shoots and roots from plants exposed to all conditions tested. Prior to drying (at 60 ºC), roots were washed thoroughly with distilled water to remove all the soil. Dried tissues were treated according to Azevedo *et al*., (2005); in brief, dried tissues were incinerated at 530 ºC during 14 h. The ashes were digested with HCl solutions and heating, followed by filtering. Ultra pure water was added until the final volume of 14 ml. Then, samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France). The real concentration of total Cr in the growing media having nominal Cr(VI) concentrations of respectively, 0; 20; 200; 1000 and 2000 mg l^{-1} was also measured by ICP-AES.

Pigments quantification

Leaf disks (0.5 cm^2) were ground in a mortal at 4 ºC with 2 ml cold Acetone/Tris 50 mM pH 7.8 buffer solution (80:20, v:v) and centrifuged at 2,800×*g* during 5 min. The supernatant was diluted to a final volume of 3 ml with additional Acetone/Tris buffer. The absorbance at 470, 537, 647 and 663 were determined with a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S Madison, MI, USA). The contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids were calculated using the formulae of Sims and Gamon, (2002). According to Lichtenthaler (1987), the molecular weights used to convert gram units to mole units were as follows: Chl *a* = 893.5 g mol^{-1}, Chl *b* = 907.5 g mol^{-1} and carotenoids = 550 g mol^{-1}. At least 5 samples (from 5 different individuals) were analyzed per condition.

Chlorophyll a fluorescence and gas exchange

Chlorophyll *a* fluorescence parameters were measured in the adaxial side of the leaf with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). Maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v/F_m = (F_m-F_0)/F_m$ by measuring the fluorescence signal from a dark-adapted leaf when all reaction centers are open using a low intensity pulsed measuring light source (F_0) and during a pulse saturating light (0.7 s pulse of 15000 µmol photons m^{-2} s^{-1} of white light) when all reactions centers are closed (F_m). Leaves were dark-adapted for 30 min using dark-adapting leaf-clips for these measurements. Following F_v/F_m estimation, after a 20-s exposure to actinic light (1500 µmol m^{-2} s^{-1}), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (15000 µmol m^{-2} s^{-1}) for 0.7 s to establish F'_m. From these measurements, quantum effective efficiency of PSII was calculated as $\Phi_{PSII} = \Delta F/F'_m = ((F'_m-F_s)/F'_m)$. Leaf gas exchange measurements were performed using a portable IRGA (LC*pro*+, ADC, Hoddesdon, United Kingdom), operating in the open mode under growth chamber conditions. Net CO_2 assimilation rate (*A*), stomatal conductance (g_s), transpiration rate (*E*) and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) were estimated from gas exchange measurements using the equations developed by Caemmerer and Farquhar (1981). The *A*/*E* ratio was used as instantaneous water use efficiency (WUE).

Gas-exchange and chlorophyll fluorescence measurements were always performed between 13:00 and 14:00 h (8 h after the beginning of the light period).

RuBisCO activity

Leaf discs of 0.5 cm^2 diameter were homogenized at 4 °C in a mortal with 1 ml extraction buffer according to Dias and Brüggemann (2010). The extraction buffer consisted of 50 mM Tris/HCl, pH 7.9, 8 mM $MgCl_2$, 5 mM Na-pyruvate, 1 mM EDTA, 2 mM K_2HPO_4, 20 mM dithiothreitol, and 0.3 % (m/v) bovine serum albumin. The homogenate was centrifuged in Eppendorf cups at 9,000×*g*. Immediately after extraction, Ribulose 1,5 bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) activity was assayed as described by Lilley and Walker (1974). This assay followed NADPH oxidation measured spectrophotometrically at 340 nm. Total activity was achieved after incubation in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min.

Soluble sugars and starch

Soluble sugars were extracted from frozen leaf discs (0.5 cm^2) with 2 ml 80 % (v/v) of ethanol at 80 ºC over 20 min (Correia *et al*., 2005). Glucose, fructose, sucrose were quantified using a spectrometric enzyme-coupled assay (Jones *et al*., 1977) and starch was quantified (Stitt *et al*., 1978) in a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S, Madison, WI USA).

Chloroplast morphology and fluorescence emission by FCM

For FCM analysis, chloroplast extraction was performed using the protocol developed by Rodriguez *et al*., (2011a). In brief, leaves were harvested as needed and ground with a mortar and pistil, in HS buffer (HEPES and sorbitol). The suspension was filtered through two 50 µm nylon meshes, and centrifuged for 2 min at 4,000 × *g*. The supernatant was placed in 2 ml of a 35 % Percoll® solution and centrifuged for 8 minutes at 1,400 × *g*. Then, the pellet was re-suspended and centrifuged twice in HS buffer at 4,000 × *g*, for 2 min. Finally, chloroplasts were analyzed in a Coulter Epics XL Flow cytometer (Beckman Coulter Inc. Hialeah FL, USA). The chloroplasts' forward light scatter (FS), corresponding to the volume of each particle and the chloroplastodial fluorescence intensity (FL) were analyzed. From the FL histogram, the events were classified in two populations (A and B) based on the level of fluorescence emitted in control plants: population B was composed by particles high FL and well defined scatters settings while population A had low FL. After this, the voltage of the cytometer and the regions characterizing these populations were kept constant for the remaining of the assay.

Data analyses and statistics

For all parameters, at least 5 replicates were analyzed and two independent experiments were performed. The results were analyzed by one-way analysis of variance (ANOVA) using the Sigma Stat program for Windows, version 3.5. Pairwise comparisons between means were evaluated by post hoc Holm-Sidak multiple comparison test.

Results

Growth, Cr and nutrient quantification

After 28 days of culture, control and Cr(VI) exposed plants showed similar survival rates (100 %). The variation in fresh and dry mass of shoot and roots is presented in Table 1. A significant decrease ($P \leq 0.05$) in fresh and dry mass was observed for tissues collected from plants exposed to 2000 mg l^{-1} Cr (VI); the reduction in shoots fresh mass was of 33 % while in roots it was of 55 % (in respect to control). As for the dry matter, the shoots from plants exposed to 2000 mg l^{-1} weighed 29 % less than control while the roots displayed a decrease in weight of 72 %, also in respect to control.

Table 1 Weight variation in shoots and roots of plants exposed to Cr. Parameters presented are the fresh mass (FM) and dry mass (DM) of shoots (s) and roots (r), being given as the mean ± standard deviation of 20 individuals. Values followed by different letters are statistically different ($P \leq 0.05$)

Cr [mg l^{-1}]	FMs (g)	FMr (g)	DMs(g)	DMr(g)
0	1.95 ± 0.606^{ab}	1.04 ± 0.253^{a}	0.15 ± 0.050^{ab}	0.14 ± 0.079^{a}
20	2.21 ± 0.363^{b}	1.16 ± 0.308^{a}	0.17 ± 0.029^{a}	0.06 ± 0.018^{b}
200	1.55 ± 0.498^{ac}	0.81 ± 0.233^{b}	0.14 ± 0.045^{abc}	0.06 ± 0.022^{ab}
1000	1.56 ± 0.659^{ac}	0.68 ± 0.304^{bc}	0.12 ± 0.051^{bc}	0.08 ± 0.084^{ab}
2000	1.30 ± 0.644^{c}	0.47 ± 0.276^{c}	0.11 ± 0.047^{c}	0.04 ± 0.017^{b}

The total Cr concentration in the control (0 mg l^{-1}) medium was below the ICPS detection limit. In other growing media with nominal concentrations of Cr(VI) of 20; 200; 1000 and 2000 mg l^{-1}, the measured values were, respectively, of 20.02 $\pm$ 0.010 mg l^{-1}; 200.03 $\pm$ 0.018 mg l^{-1}; 1000.04 $\pm$ 0.036 mg l^{-1}; 2000.09 $\pm$ 0.080 mg l^{-1} of total Cr.

Total Cr accumulation in both tissues is presented in table 2. In roots, the level of Cr accumulated increased ($P \leq 0.05$) with the increase of Cr(VI) dosages. The difference in accumulation was as high as 51 fold, between control and roots exposed the highest concentration tested. In shoots, all Cr(VI) treatments caused a significant increase in the amount of Cr accumulated ($P \leq 0.05$), despite that little difference could be observed between 20 and 200 mg l^{-1}. Exposure to 1000 and 2000 mg l^{-1} increased Cr levels, with the maximum difference to control being of 26 fold (at 2000 mg l^{-1}). At any rate, the major difference in the level of Cr accumulation between tissues of the same plant (condition) was of almost 4 fold, for plants exposed to 200 mg l^{-1}, while for all the remaining exposures this difference ranged between 2 and 2.5 fold.

Table 2 Total Cr accumulated (Cr) in shoots and roots exposed to different Cr concentration. Values are given as the mean ± standard deviation of the mean. Values followed by different letters are statistically different ($P \leq 0.05$)

Cr (mg l^{-1})	Shoots (µg g^{-1} DM)	Roots (µg g^{-1} DM)
0	19.2 ± 1.42^{a}	24.0 ± 17.61^{a}
20	122.2 ± 52.09^{b}	295.5 ± 107.88^{b}
200	137.5 ± 26.10^{b}	538.0 ± 78.79^{c}
1000	350.2 ± 75.58^{c}	733.0 ± 135.62^{d}
2000	529.5 ± 68.11^{d}	1236.8 ± 125.34^{e}

The effect of Cr(VI) exposure in some nutrients accumulation and partition is displayed in table 3. In roots, Cr(VI) significantly decreased ($P \leq 0.05$) the amount of P accumulated; contrarily, the levels of Zn and Fe increased significantly in response to Cr(VI) ($P \leq 0.05$). As for leaves, Mg and Cu levels, which in roots suffered little alteration ($P > 0.05$), increased in shoots exposed to Cr(VI) (around 35 % higher for Mg, 50 % higher for Cu) ($P \leq 0.05$). The elements P, Zn, and Fe presented in shoots, the same trend observed in roots exposed to Cr: decrease in P content and increase in the remaining elements.

Table 3 Nutrient accumulation in roots and shoots of plants exposed to Cr. Values are given as the mean ± standard deviation of the mean. Values followed by different letters are statistically different ($P \leq 0.05$)

	Cr(mg l^{-1})	Mg(mg g^{-1})	P(mg g^{-1})	Zn(µg g^{-1})	Cu(µg g^{-1})	Fe(mg g^{-1})
Roots	0	2.7±0.10^{a}	11.6±1.31^{a}	114.9±16.7^{a}	38.7±8.94^{a}	0.23±0.050^{a}
	20	3.6±1.40^{b}	10.4±3.90^{a}	212.8±10.91^{b}	50.0±7.70^{a}	2.30±1.54^{bc}
	200	1.5±0.18^{c}	6.9±1.04^{b}	247.9±64.81^{b}	57.7±39.63^{a}	1.60±0.32^{b}
	1000	2.7±0.99^{ab}	7.0±0.16^{b}	172.1±25.52^{c}	45.3±16.35^{a}	2.52±1.33^{bc}
	2000	1.8±0.26^{ac}	5.7±0.28^{b}	156.1±18.04^{c}	58.8±26.28^{a}	3.54±0.76^{c}

	Cr(mg l^{-1})	Mg(mg g^{-1})	P(mg g^{-1})	Zn(µg g^{-1})	Cu(µg g^{-1})	Fe(mg g^{-1})
Shoots	0	1.6±0.10^{a}	8.6± 0.32^{a}	37.9±3.40^{a}	11.2±2.58^{a}	0.15±0.037^{a}
	20	2.3±0.51^{bc}	6.9±0.36^{b}	63.0±1.85^{b}	14.3±5.80^{ab}	0.54±0.350^{a}
	200	2.2±0.30^{b}	6.0±1.16^{c}	68.1±5.17^{b}	13.0±1.82^{a}	0.60±0.153^{a}
	1000	2.8±0.42^{c}	7.5±0.59^{b}	80.7±7.74^{c}	17.7±3.58^{b}	1.46±0.55^{b}
	2000	2.4±0.32^{bc}	4.2±0.58^{d}	62.3±8.00^{b}	23.2±0.58^{c}	2.12±0.55^{c}

Gas-exchange parameters

The gas exchange status of plants exposed to Cr(VI) displayed significant changes in all the biomarkers measured, being more significant at higher doses. Figure 1 presents the variation of *A* with the increase in Cr(VI) concentration: *A* decreased from control to 20 and 200 mg l^{-1} ($P \leq 0.001$), followed by a stabilization at 1000 and 2000 mg l^{-1} (not significantly different from 200 mg l^{-1}; $P > 0.05$). The lowest value was observed at 2000 mg l^{-1}, representing a decrease of 84 % in respect to the values detected in control.

Changes in WUE of plants exposed to Cr(VI) were highly similar to what was observed for *A* (Figure 1). Significant differences to control were observed starting at the lowest concentrations used ($P \leq 0.001$). Unlike *A*, however, the lowest value observed for WUE was in plants exposed to 200 mg l^{-1}, which correspond to a decrease of 79 % of what was observed in control plants.

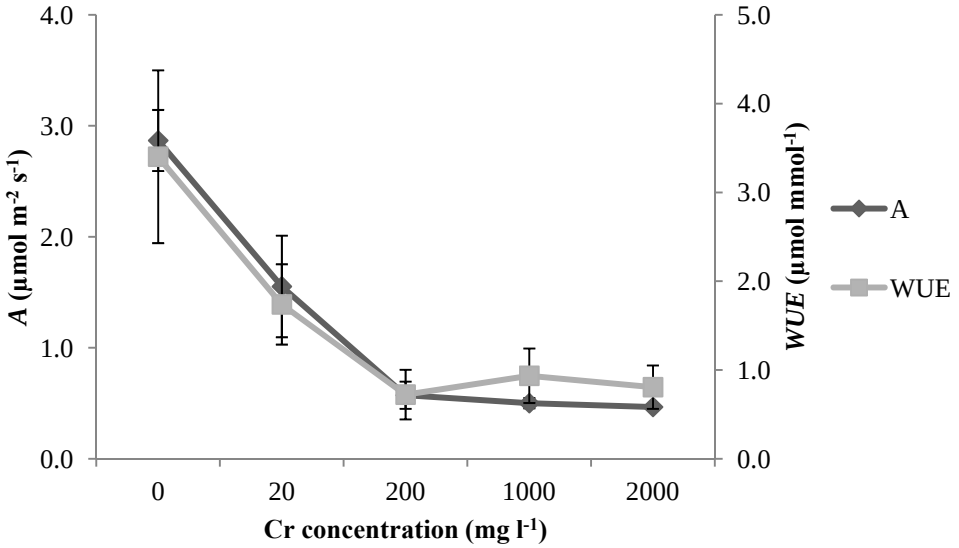

Figure 1 Variation of the photosynthetic rate (*A*) and water use efficiency (WUE) of pea plants exposed to Cr. Values are given as mean and standard deviation of the mean. For both parameters, all values were statistically significant from the control ($P \leq 0.05$).

Regarding the plot for *E* and g_s (Figure 2), it can be observed that both markers present a similar response to Cr(VI). A decrease in both parameters was visible starting at 200 mg l^{-1}, which is still not significantly different from control. The decrease continues for plants exposed to 1000 mg l^{-1} ($P \leq 0.001$) being that the values observed at 2000 mg l^{-1} are not significantly different from those at 1000 mg l^{-1} ($P > 0.05$). The maximum decrease in both parameters was observed at different concentrations though; while for *E* a decrease of 37 % was observed for plants exposed to 2000 mg l^{-1}; the lowest g_s was detected in response to 1000 mg l^{-1} of Cr(VI) (44 % decrease in respect to control).

Unlike all the other biomarkers so far, the C_i/C_a of control plants presented the lowest value and Cr(VI) exposure caused an increase in this parameter. Differences from control values were significant for plants exposed to 200 mg l^{-1} and higher doses ($P \leq 0.001$) (Figure 2). The highest variation observed was from control to 200 mg l^{-1} exposed plants, with an increase of 9 % from the former to the latter.

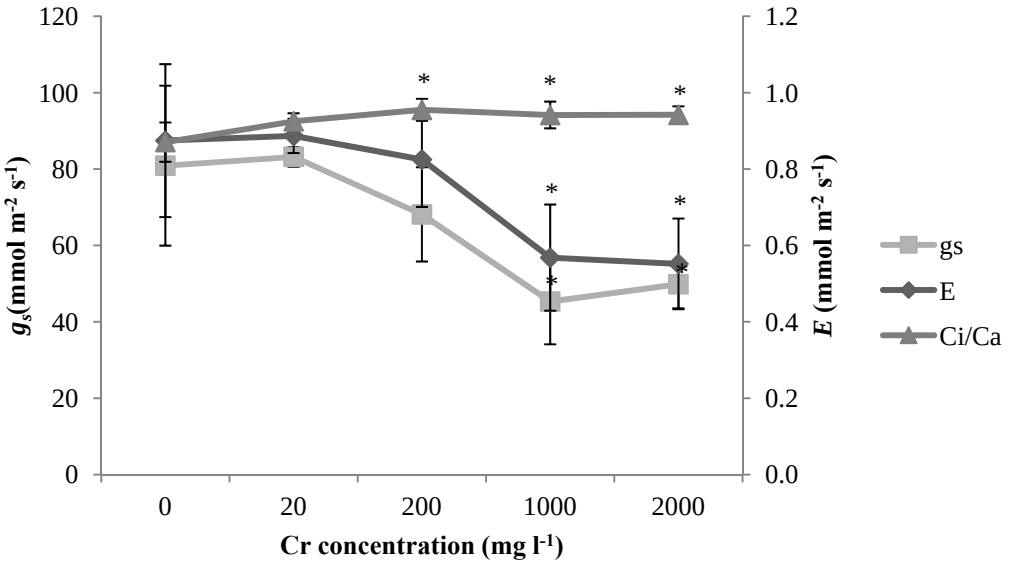

Figure 2 Transpiration rate (*E*), stomatic conductance (g_s) and intra cellular to atmospheric CO_2 ratio (*Ci/Ca*) of pea plants exposed to given concentration of Cr. Values are mean and standard deviation of the mean. Points marked by an (*) are statistically significant from the control ($P \leq 0.05$).

Chlorophyll and carotenoids

The pigment quantification revealed that Cr(VI) exposure induced, in general, an increase in content (Figure 3). The increase was statistically significant at 1000 mg l^{-1} and 2000 mg l^{-1} for all pigments ($P \leq 0.001$) but at 200 mg l^{-1}, Chl *b* and carotenoids levels were already significantly higher than control ones. The maximum increase in pigment content was of 50 % in Chl *b* (2000 mg l^{-1}); 40 % in carotenoids (1000 mg l^{-1}) and 38 % in Chl *a* (2000 mg l^{-1}).

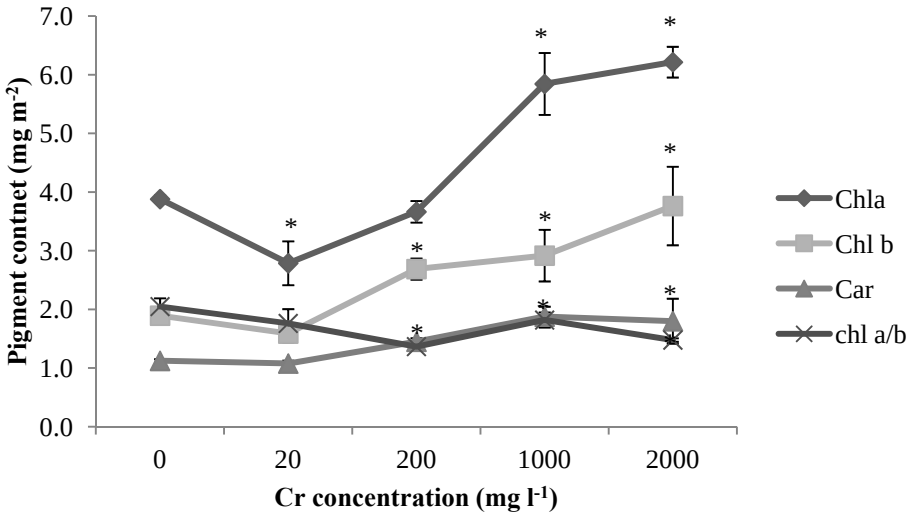

Figure 3 Pigment content variation of leaves from plants exposed to Cr. Pigments are Chlorophyll a (Chl *a*) Chlorophyll b (Chl *b*) and Carotenoids. The Chla/Chlb ratio is also given (Chl *a*/*b*). Data is presented as mean and standard deviation of the mean. Points marked by an * are statistically significant from the control ($P \leq 0.05$).

Chlorophyll a fluorescence

The analysis of Chl *a* fluorescence-related biomarkers showed that Cr(VI) interferes with these parameters (Table 4). The F_v/F_m of control plants, 0.73 ± 0.004 (mean ± sd), was significantly higher than that of plants exposed to 200 mg l^{-1} (0.59 ± 0.003; $P \leq 0.001$) and 2000 mg l^{-1} (0.59 ± 0.003; $P \leq 0.05$).

The determination of Φ_{PSII} indicated that Cr(VI) caused a significant decrease of this biomarker in plants exposed to the maximum dose ($P \leq 0.05$). The decrease observed from control (0.86 ± 0.061) to 2000 mg l^{-1} (0.74 ± 0.030) represents a drop of 14 % in Φ_{PSII}.

Table 4. Chlorophyll *a* fluorescence parameters measured in control and Cr-exposed plants. Values given are the mean ± standard deviation. Parameters presented are the maximum quantum efficiency of photosystem II (F_v/F_m) and the effective quantum yield of PSII (ΦPSII).

Cr (mg l^{-1})	F_v/F_m	ΦPSII
0	0.73 ± 0.04^{a}	0.86 ± 0.06^{a}
20	0.70 ± 0.04^{a}	0.85 ± 0.06^{a}
200	0.59 ± 0.03^{b}	0.87 ± 0.06^{a}
1000	0.68 ± 0.03^{a}	0.84 ± 0.06^{ab}
2000	0.59 ± 0.02^{b}	0.74 ± 0.03^{b}

FCM evaluation of chloroplast morphology and fluorescence

The evaluation of FS, FL and % of intact chloroplast extracted from control and Cr(VI) exposed plants revealed that the latter significantly affected all the biomarkers assessed (Table 5). Starting with chloroplast's morphology, the FS of chloroplasts from plants exposed to Cr(VI) was at all times lower than control values. Moreover this decrease was significantly different for all conditions ($P < 0.05$) but 200 mg l^{-1} ($P = 0.124$). The lowest FS was observed in chloroplast extracted from plants exposed to 2000 mg l^{-1}, with 30 % less volume than control ones.

Cr(VI) exposure caused significant variation of chloroplasts' FL, in plants treated with to 200 mg l^{-1} and 2000 mg l^{-1} (in respect to control). While chloroplasts from plants exposed to 200 mg l^{-1} had higher FL ($P < 0.001$), the FL from plants exposed to 2000 mg l^{-1} was 25 % lower than the control's mean value ($P < 0.001$). Finally, the percentage of intact chloroplasts was also

affected by Cr(VI); the percentage of intact chloroplast observed in control and plants exposed to 20 mg l^{-1} was around 80 %. This value significantly decreased starting at 200 mg l^{-1} ($P < 0.05$), and presenting the lowest value at 1000 mg l^{-1} (45 %).

Table 5 Flow cytometric evaluation of structural and functional status of chloroplast extracted from control and Cr-exposed plants. Values are presented as mean ± standard deviation of the mean. Parameters are volume (FS), fluorescence emission (FL) and percentage of intact chloroplast (%). Values followed by the different letters are statistically different ($P \leq 0.05$)

Cr (mg l^{-1})	FS	FL	%
0	55.5 ± 4.02^{a}	257.8 ± 4.61^{ac}	83.0 ± 5.30^{a}
20	44.5 ± 2.10^{bd}	251.9 ± 6.01^{a}	79.0 ± 10.20^{a}
200	51.0 ± 1.70^{ac}	284.0 ± 2.60^{b}	50.0 ± 4.57^{b}
1000	48.2 ± 1.22^{c}	264.1 ± 3.82^{c}	44.6 ± 1.86^{b}
2000	39.3 ± 1.91^{d}	194.8 ± 4.88^{d}	58.4 ± 3.61^{b}

Calvin cycle enzymes

Cr(VI) provoked a noxious effect in RuBisCO activity; except for plants exposed to 20 mg l^{-1} all other treatments caused a significant decrease in the enzyme's activity ($P \leq 0.001$). Plants exposed to 200 mg l^{-1} presented a plunge of 2.5 fold in respect to control, about the same of what was verified for plants exposed to 1000 mg l^{-1}. The activity of RuBisCO was even lower in plants exposed to the highest concentration of Cr(VI), being the decrease in activity of 90 % in regard to control plants (Figure 4), which was significantly lower than the values observed for all the other concentrations.

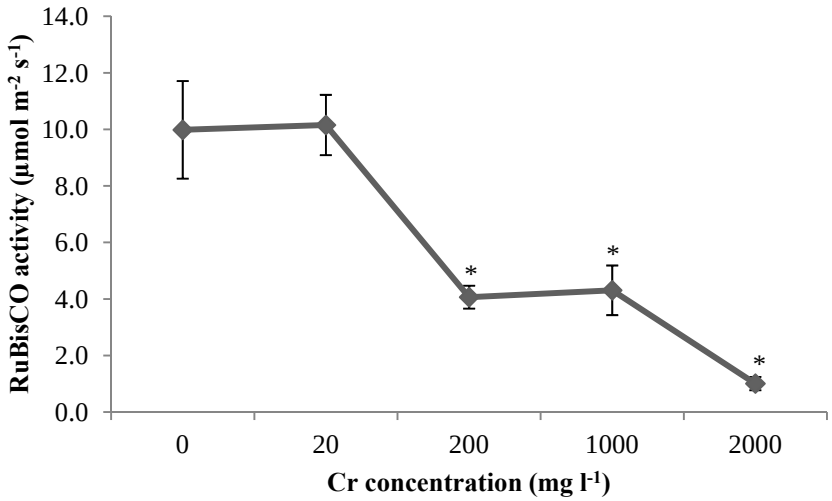

Figure 4 Maximum RuBisCO activity of leaves from control and Cr exposed plants. Values are given as mean and standard deviation of the mean. Points followed by (*) are statistically different from the control ($P \leq 0.05$).

Soluble sugars and starch

Quantification of soluble sugars and starch indicated that, as a general trend, they increase when the concentration of the pollutant increases (Figure 5). Starting with glucose, exposure to Cr(VI) caused a continuous increase of this sugar levels, from control up to 1000 mg l^{-1} ($P \leq 0.001$). At 2000 mg l^{-1}, the level of sucrose decreased sharply when compared to 1000 mg l^{-1} but it was still significantly higher than what was observed for control ($P \leq 0.001$). The amount of glucose in plants exposed to 1000 mg l^{-1} was 82 % and 41 % higher than what was detected in control and 2000 mg l^{-1}, respectively. Fructose levels significantly and continuously increased with increasing concentrations of Cr(VI) ($P \leq 0.001$). At 20 mg l^{-1} the amount of fructose had raised 37 % (from control), peaking in plants exposed to 2000 mg l^{-1} (76 % higher than control). The effect of Cr(VI) in sucrose levels was somewhat more heterogeneous and the only treatment that caused significant variation ($P \leq 0.001$) from control was 1000 mg l^{-1} (30 % more sucrose than control).

Relatively to the starch content, only at 1000 and 2000 mg l^{-1} of Cr(VI) produced a significant increase in the amount of starch accumulated ($P \leq 0.001$), near 35 % more than control plants.

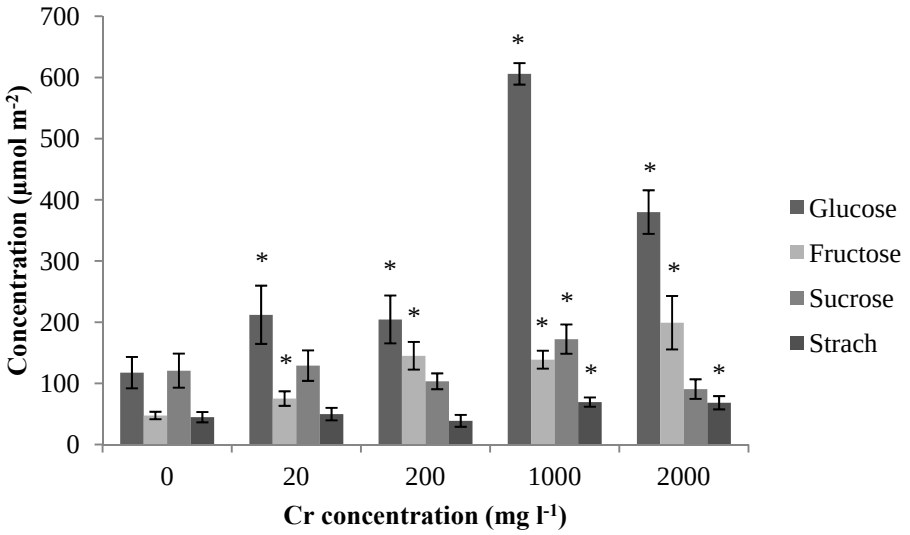

Figure 5 Cr effect in the soluble sugars and starch content of leaves from control and exposed plants. Soluble sugars measured are glucose, fructose and sucrose. Values are presented as mean and standard deviation of the mean. Bars marked by (*) are statistically different from the control ($P \leq 0.05$).

Discussion

Cr and Nutrient accumulation

Cr, due to its complex mechanism of speciation, uptake and intracellular interaction is likely to behave, at least at some extent, differently from other metals. It is believed (Shanker *et al*., 2005, Cervantes *et al*., 2001) that Cr is transported by carriers used for the uptake of essential metals of plant's metabolism (*e.g*. Fe, S and P) and therefore, might compete with these elements. Our results indicate that the level of P decreased with the increase of Cr in both roots and shoots. Moreover, in roots but not in shoots, the level of Cr accumulated presented a significant negative correlation with the amount of P (P = 0.034; r = -0.905), being in agreement with the current hypothesis of these elements competing for transport. Fe on the other hand showed a positive correlation with Cr in both roots (P = 0.0257; r = 0.922) and shoots (P = 0.0000034; r = 1.000) indicating that, at least in these conditions, the uptake of Fe is enhanced by Cr exposure, by still unknown mechanisms.

Gas exchange parameters and RuBisCO activity

Cr has been shown to have harmful effects on A, E and g_s in *L. perenne* (Vernay *et al*., 2007). This is in agreement with the data presented in our investigation for pea plants exposed to Cr(VI): increasing metal concentration induced a reduction of A, E and g_s and also an increase in

C_i/C_a. The increased C_i/C_a values observed in Cr(VI) exposed plants suggest that the decrease in g_s and also in *E* did not affect the *A*. To reinforce this idea, the Pearson's correlation between g_s and *A* of our Cr(VI) treated plants shows that with a $P = 0.126$, the probability of these parameters being correlated is highly unlikely. Moreover, the Pearson's correlation between *A* and C_i/C_a demonstrated a strong and negative correlation between these parameters ($P = 0.00715$, r= -0.967), indicating that the decrease in *A* is also not due to low abundance of CO_2. Vernay *et al*., (2007) hypothesized that the decrease in *A* might have been due to the inhibition of RuBisCO. In the present work, we found hard evidence of this, as the low consumption of CO_2 (stable C_i/C_a for high Cr(VI) concentration) and the decrease of RuBisCO's activity (with a decrease of 90 % of the enzyme's activity at the maximum Cr dosage) point to a decrease of *A* due to a reduction of RuBisCO activity. A possible justification to the decrease in RuBisCO activity might be related with the local substitution of Mg by Cr, decreasing the enzyme's affinity to CO_2. This has been reported with exposure to other metals (Van Assche and Clijsters, 1990) and it is a fitting explanation for the results obtained.

Another biomarker significantly affected by Cr(VI) exposure was WUE, which presented a very similar pattern to *A* ($P = 0.000873$, r = 0.992). This data demonstrates that Cr(VI) critically affects the ability of plants to regulate the rates of water loss (*E*) associated with the photosynthetic rate.

Pigment content

It has been demonstrated by several authors that Cr exposure can affect (in general decreasing) the amount of pigment in leaves of several species (Redondo-Gómez *et al*., 2011, Subrahmanyam, 2008, Vernay *et al*., 2007). In our experiments, Cr induced an increase of some of the pigments analysed, in respect to control. Carotenoids play a key role in protecting chlorophyll pigments under stress conditions (Choudhury and Behera, 2001), which may justify its increase in Cr exposed plants. Moreover, we also observed a significant increase in the accumulation of Mg, Fe and Zn in shoots of plants exposed to Cr. Mg and Fe are directly involved in chlorophyll biosyntehsis: Mg is at the center of the chlorine ring of chlorophyll molecules and Fe is needed for chlorophyll biosynthesis (Barcelo *et al*., 1985). Zn on the other hand has been proven to alleviate the negative effect of heavy metals (Cd) to chloroplast and its photochemical function (Aravind and Prasad, 2004). The increase in the accumulation of these nutrients might be part of a strategy to minimize the negative effects of Cr to leaves pigments by increasing chlorophyll synthesis (Fe and Mg) and protecting it from stress (Zn). At any rate, and despite the increase in pigment content, we observed a significant decrease in the Chl *a*:*b* ratio of all but 1000 mg l^{-1} treated plants. A decrease in this ratio is considered to be a symptom indicating stress (namely oxidative) conditions (Rout *et al*., 1997, Pandey *et al*., 2009). This data also reflect that Chl *a* was more affected by Cr(VI) exposure than *b*.

Chlorophyll a fluorescence

Cr(VI) also proved to have deleterious effects in the levels of fluorescence emitted, as indicated by the FL, F_v/F_m and Φ_{PSII}. These biomarkers coincide that at 2000 mg l^{-1} there is a significant impairment of fluorescence emission, this together with the pigments' content data suggest that, at this concentration, the photochemical apparatus might have been compromised even if the amount of pigments on those plants were significantly higher than that of control. So, being that the amount of chlorophyll increased but fluorescence emission decreased we hypothesize that the deleterious effects of Cr had other target than pigment synthesis. In fact, some of the parameters assessed by FCM indicate that chloroplast integrity might have been compromised as a result of Cr(VI) exposure, namely the significant decrease in volume (for all concentrations) and the significant reduction in the number of intact chloroplasts (near 30 % in respect to control) observable starting at 200 mg l^{-1}. Rodriguez *et al*., (2011a) observed that, as time progressed, chloroplast extracted from plants exposed to Paraquat suffered a series of transformations in volume (correlated with a decrease of FS), function (FL) and integrity, similar to those reported here. Pandey *et al*., (2009) commented that despite that they have found a decrease in F_v/F_m (altogether with a decrease in chlorophyll content), in other works (Dhir *et al*., 2008), Cr stimulated PSI's activity. They hypothesize, based on the information of Makino *et al*., (2002) that this might have been an acclimation response to cope with the additional flow of electrons during stress. If this is the case, it is likely that what was observed in our work was a stimulation of PS activity (which then translates on higher FL) up to the levels of Cr accumulated in leaves of plants exposed to 200 mg /l. At higher dosages, an inhibition of electron activity occurred (The FL observed at 1000 mg l^{-1} is significantly lower than that of 200 mg l^{-1} but higher than 2000 mg l^{-1}).

Soluble sugars and starch

The amount of soluble sugars and starch accumulated in leaves were also used as endpoints of Cr(VI) toxicity, being that these are some of the principal end products of photosynthesis and are highly sensitive to environmental stresses (Rosa *et al*., 2009). On an extensive review, Rolland *et al*., (2006) described the role of sugars as stress sensors and as key players in metabolism and homeostasis regulation, highlighting the central roles of sugar signals and signaling in plant life.

Our results demonstrated that Cr caused heterogeneous effects on the soluble sugars accumulation, with different trends for glucose, fructose and sucrose. Fructose presented a significant and positive correlation with the level of Cr accumulated in the leaves (P = 0.047; r = 0.882); likewise, it also presented a significant and negative correlation with A (P = 0.0461; r = -0.893) and RuBisCO activity (P = 0.0031; r = -0.981). Taking into account the role that this sugar might have as putative signalling molecule, it is probable that the increasing levels of fructose

observed acted as part of the signalling cascade leading to the down-regulation of RuBisCO activity and consequent decrease in the *A*. The positive correlation with the level of Cr accumulated on leaves is also congruent and strengthens the putative role of fructose as signaling molecule, in a negative feedback loop.

The levels of glucose and sucrose both coincide in that they peaked with exposure to 1000 mg l^{-1} and at 2000 mg l^{-1} there was a significant plunge in these sugars content. This decrease might be related with the near stoppage of the photosynthetic activity observed at this 2000 mg l^{-1}. High sugar levels (observed in our plants treated up to mg l^{-1}) cause the up regulation of storage, a sink activity (Rolland *et al*., 2006), which contrast with the photosynthetic role that leaves should carry. The significant increase of starch observed at the two highest exposures dosages corroborates, together with the decrease in all the other photosynthetic parameters that, leaves passed from photosynthetic to storage activity.

Fresh and dry weight

Like verified in other reports (*e.g.* Subrahmanyam, 2008), we also observed a pronounced decrease in both fresh and dry matter weight, in response to Cr exposure. This fact is in agreement with most of the results that we obtained: a decrease in several parameters (most importantly in *A*) justifies the decrease in DM observed. Unlike what was observed by those authors in wheat, the reduction in root's DM was significantly higher than in shoots (74 % against 29 % respectively), despite the fact that the root/shoot Cr accumulation ratio was higher in wheat (5 - 7 fold) than in our pea plants (2 - 3 fold). This difference in performance might indicate that even though pea accumulated higher levels of Cr in leaves than wheat, the latter is more sensitive to this metal.

Environmental perspective

The data that is provided in this article gains even higher relevance if the EU maximum admitted dosage for total Cr in agricultural purpose water and the levels of Cr(VI) that can be observed in contaminated sites are factored in. The maximum admitted level in agricultural purpose waters, for total chromium, is 20 mg l^{-1}, and in some cases is even stated that due to the lack of information about Cr toxicity, more restrictive values should be recommended. Our data indicates that while most of the endpoints were only significant starting with exposure to 200 mg l^{-1}, exposure to 20 mg l^{-1} of Cr(VI) significantly affected some of the biomarkers tested (i.e. *A*; WUE and sugar contents) and thus, it might be advisable to propose more restrictive values. As for the highest dosage used, Cr (VI) content in waste waters coming from tannery industries can rise up to 5000 mg l^{-1} and even though we did not use such a extreme concentration, our data demonstrate that starting at 200 mg l^{-1} and peaking at 2000 mg l^{-1}, Cr(VI) causes severe damage to the photosynthetic status of the plants.

Conclusions

In conclusion, results demonstrate that all the parameters analyzed were affected by Cr but the ones regarding the biochemical aspects of photosynthesis were more affected than the photochemical efficiency. We also confirm that FCM can be successfully used to detect Cr-related effects in chloroplast, in what is an innovative application of this technique to plant phytotoxicity.

Regarding that data for Cr accumulation and phytoremediation is scarce (Cervantes *et al*., 2001, Diwan *et al*., 2008), is important to highlight that our pea plants accumulated high levels of Cr in roots and even translocated significant amounts to the shoots. Moreover and despite that they can't be classified as hyper accumulators, pea produces more biomass than some of the proposed species for phytoremediation (*e.g. Brassica juncea*) which might justify potential applications of this species in Cr phytoremediation strategies.

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CHAPTER IV

CONCLUSIONS AND FINAL REMARKS

Conclusions and Final Remarks

Since the moment mankind shifted from being a nomadic hunter-gatherer society to settling in favorable locations and develop agriculture and pastoralism, the impact of Human actions on the environment increased dramatically.

With the development of mining and metal ore smelting, the first big step for metal contamination was given. Since then, the application of metals has continuously raised and concomitantly, metal poisoning and environmental pollution have also increased, becoming a major concern for modern societies.

Plants, which are one of the most important resources of mankind, providing constant supply of food and products, are among the organism more exposed to pollution, due to their lack of a flight response when confronted with danger. Being this, understanding the mechanism by which metals induce phytotoxicity and the effects of metals exposure is imperative.

In this context, the aim of the investigations composing this thesis was to evaluate the phytotoxicity of two of the most toxic metal contaminants, Cr and Pb, using as a model one of the major crop species, *Pisum sativum* L.

As referred in chapter I, Cr genotoxicity have been studied in humans and animals but in plants, information is scarce and mostly based on cytogenetic evaluation of DNA damage. Pb phytotoxicity, despite being more studied than Cr's, is still enveloped in murky background; the mechanism(s) of action by which this metal induced genotoxicity is still unclear even in humans and animals (Beyersmann and Hartwig, 2008, García-Lestón *et al*., 2010).

Chapters II, III and IV were dedicated to study the effects and mechanism of Pb and Cr induced genotoxicity. In these chapters, an approach was used to evaluate different types of DNA damage, by applying FCM and Comet assay, which assess the accumulation of clastogenic damage (FPCV) and DNA strand brakes (Tail DNA % and Tail Moment). The amount of DNA per somatic cell and DNA ploidy level of pea was also estimated as well as the dynamics of cell cycle. The results presented in these chapters indicate that both techniques provided accurate and sensitive biomarkers of the DNA damage endpoint, detecting significant changes in both roots and leaves of plants exposed to Pb and Cr. The level of DNA damage observed in roots was significantly higher than that of the leaves. Roots had direct contact with the metals and it is known that in most cases, this organ acts like a barrier against metal translocation, which might justify why this organ accumulated significantly higher levels of Cr and Pb than leaves the higher level of DNA damage observed in roots. The difference between Cr and Pb accumulation in both tissues was interesting: taking the highest dosage used, while roots accumulated more Pb than Cr (3500 μg Pb g^{-1} DW, 1300 μg Cr g^{-1} DW), for shoots the opposite was observed (530 μg Cr g^{-1} DW, 110 μg Pb g^{-1} DW). In fact, while the maximum difference in metal accumulation between tissues was of 37 fold for Pb, for Cr the highest difference was of 4 fold. These results are illustrative of the different interaction between the plant and these metals: Pb seems to be sequestered in the roots and presents little mobility once inside the plant; Cr on the other hand moves more freely from roots to shoots.

The differences between metals were not restricted to accumulation, the evaluation of DNA strand brakes (by Comet assay) gave substantially different results depending on the metal: Cr exposed plants presented a dose-response increase in damage but for Pb-treated plants this was not verified; plants exposed to 1000 mg l^{-1} presented significantly higher levels of damage than plants exposed to 2000 mg l^{-1}. The difference observed between both metals at this level fit with the current hypothesis explaining the mechanism of action of both metals; while Cr directly binds to DNA to form adducts, Pb does not interfere directly with DNA but promotes the formation of DNA-DNA and DNA-protein links which decrease migration in the Comet assay. This pattern was also observed by Wozniak and Blasiak (2003), Rucinska *et al*. (2004), Gichner *et al.* (2008) and working with tobacco, lupin and human lynphocytes.

Relatively to the cell cycle dynamics, exposure to the maximum dose of both metals caused cell cycle arrest at the G_2/M transition checkpoint. An arrest of the cell cycle at this checkpoint occurs when DNA synthesis has been compromised, to give cells extra time to either repair the damage (O'Connell and Cimprich, 2005) or activate an apoptosis-like program. In some cases though, cells might continue with proliferation without completing the damage repair (Carballo et al., 2006). From the data presented in chapters II and III, it is evident that there is a link between DNA damage and cell cycle arrest at the highest dosage, which also correlated to a decrease in growth observed in roots of plants exposed to those concentrations of heavy metals. Moreover, the evaluation of MSI helped to explain why, despite that significant DNA damage was detected in lower dosages, only at the maximum dosage an arrest at the cell cycle was observed, since signs of MSI could only be observed at that dosage. Also, roots of plants exposed to 2000 mg l^{-1} showed evidence of polyploidization, with a secondary population of cells presenting a tetraploid cytotype, which is agreement with the cell cycle data.

A final comparison of the genotoxic effects of both heavy metals in pea indicates that Cr is more genotoxic than Pb, inducing higher mutation frequency and polyploidization despite that the amount of Cr accumulated in roots was about half of the amount of Pb accumulated. From these conclusions, a model of Cr/Pb genotoxicity mechanism was developed, which is in agreement with what is kwon for animals, in particular for humans. The model outlines that metal toxicity, after inducing a critical level of DNA damage, leads to malfunction of the DNA repair system, which might in turn induce problems with the cell cycle/division machinery, causing arrest and in extreme cases, polyploidization.

On the technical side, the suitability and sensitivity of the Comet assay and FCM to evaluate genotoxic-related endpoints was evaluated and it was shown that: The Comet assay allows to detect DNA damage at lower dosages than FCM but the type of information given by these techniques is complementary rather than redundant and the application of both techniques in this type of assays is highly recommended.

The mechanics and effects of metal's interference with the photosynthetic processes have been better characterized than genotoxicity. Due to the fact that metal-induced toxicity at the photosynthetic level has been a target of investigations for a long time, most of the methodologies

used are well established but innovation and the development of new methodololgies/markers are always welcomed.

Chapter III presented the optimization of FCM to study the chloroplast's morphology and functional status, in plants exposed to Paraquat, a classical inhibitor of PSII electron transporter chain. Few reports have tried to apply FCM's potential to study chloroplast and there are even less reports focused on evaluating the effects of hazardous substances in these organelles. In the investigation reported in chapter III, the volume and granularity of chloroplast extracted from plants exposed to paraquat proved to be an excellent indicator of herbicide toxicity. Furthermore, a comparison between FCM, PAM fluoromentry and pigment content was also carried, in order to assess if chloroplast auto-fluorescence emission, as measured by FCM, related to those classical techniques. Results demonstrate that FCM and PAM fluorometry present a strong positive correlation value; even though FCM measurements were performed on isolated chloroplasts while for PAM fluorometry, intact leaves (i.e. still part of the plants) were used. The main conclusion of chapter III-1 is that FCM is suitable for evaluating the structural and functional status of stressed chloroplast, providing new markers for this type of studies. The potential of FCM to study photosynthetic related processes was also confirmed.

Chapters III-2 and III-3 focus on the effects of Pb and Cr in the photosynthetic capability of pea plants. The results obtained suggest that the reduction of the photosynthetic rate in Pb exposed plants were mainly due to biochemical process *e.g*. decreased RuBisCO activity. Results also indicate that the photochemical apparatus seems to be very resistant to Pb toxicity because the pigment content and fluorescence endpoint were seldom affected by exposure to Pb. Cr on the other hand proved to be significantly more toxic than Pb, for most of the photosynthetic endpoints. Like in plants exposed to Pb, Cr treatment was more aggressive to the gas exchange, biochemical and chloroplastidial morphology markers assessed than to those related to the photochemical apparatus. However, contrary to the evidence found with Pb treatments, exposure to Cr (at least at the highest dosages) induced significant negative effects on the photochemical apparatus, proving that despite having some degree of resistance to metal toxicity it can still be damaged by Cr dosages within the range tested. This might not be surprising if metal accumulation is taken into account; Cr content on the leaves several fold higher than Pb; even though these metals have different mechanisms and toxicity targets, the difference in accumulation might justify why Cr induced more damage than Pb.

As a summary of this thesis, the questions posed in chapter I were properly responded, it was demonstrated that both metals induced genotoxicity: DNA strand brakes and clastogenicity, cell cycle arrest and MSI. In Cr(VI) treated plants it was even possible to observe evidence of polyploidization.

Evidence of Pb and Cr deleterious effect at the photosynthetic level were also observed, being that the respiration and biochemical processes were more affected than the photochemistry apparatus. Moreover, the evaluation, by FCM, of the chloroplast's structural and functional status proved to be a good indicator of metal toxicity and a valuable tool for future investigations.

It is very important to highlight that the lowest dosage used for both metals (20 mg l^{-1}), which was based on the maximum admitted levels in agricultural purpose water in the Portuguese legislation (also based on EU directive), significantly affected some of the genotoxicity and photosynthetic biomarkers. These results are indicative that even at such low dosages, Pb and Cr are capable of hampering the plant's photosynthetic capabilities and damage the DNA and thus, more restrictive values should be recommended.

Future Perspectives

Metal pollution is a major concern and even with the advances that had occurred in the latter years, there is still a long way to run until we understand the inherent mechanism and totality of effects these pollutants cause to plants.

- The link between DNA damage and cell cycle arrest observed in our metal exposed plants opens the question of how is the cell cycle machinery affected and which regulatory pathways lead to the cytostaticity observed. MAPK and cyclins are among the most interesting candidates in this situation, as changes in the expression level of these proteins or in their activity could justify the results observed.
- The DNA single and double strand brakes observed by Comet assay and the concomitant MSI indicates that the DNA repair system might be compromised. It would be interesting to assess the DNA repair capacity of plants exposed to these metals (by performing an exposure plus recovery assay) and also to understand why the DNA repair system failed. Putative candidates could be either on the side of DNA damage recognition (on the cell cycle checkpoint, though the arrest on the cell cycle observed suggests that, at least at some extent, this system is remains functional) or in the repair mechanism (*e.g* DNA ligase, topoisomerase, glycosylase, Nucleotide excision repair enzymes, plant homologs of Mut proteins). In humans, defective Mut proteins have been shown to affect genome stability which is linked to MSI and thus off all the candidates, this might be the most appealing to start with.
- Performing a genome-wide expression analysis (cDNA-AFLPs) would be very useful in order to further dissect the interaction and responses of these plants when exposed to metals.
- On an environmental perspective, the potential of *P. sativum* for phytoremediation of metal polluted soils deserves further evaluation. In this trail, it would be important to evaluate the toxicity of more complex mixtures of metal pollutant, like the ones located in contaminated sites or by using industrial wastes.

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